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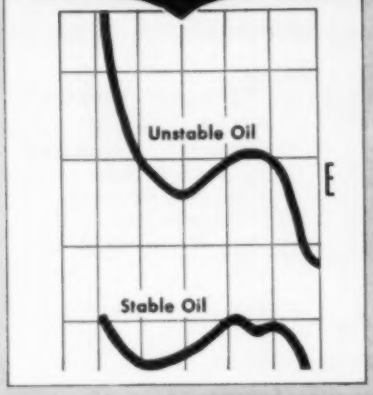

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVI — *August 1957* — NUMBER 8 — CONSECUTIVE NO. 16

The Effect of Reserpine and Meprobamate on Gastric Acid Secretion*

By G. VICTOR ROSSI, HAROLD CAVNER, and JOSEPH W. E. HARRISSON†

Reserpine administered either orally or subcutaneously in a dose of 0.5 mg./Kg. did not appreciably alter the volume of gastric fluid secreted by rats during a four-hour period after pyloric ligation, but significantly increased both free and total titratable acidity. Subcutaneous injection of 0.5 mg./Kg. of oxyphenonium markedly reduced the volume of gastric fluid secreted following subcutaneous injection of reserpine, but did not decrease the titratable acidity per ml. of gastric juice. Oral administration of 15 mg./Kg. of meprobamate also increased free and total titratable acidity in the pyloric ligated rat. Intramuscular injection of 1.0 mg. of reserpine or 20.0 mg. of meprobamate markedly increased titratable free acidity in dogs prepared with gastric fistulas. The effect on gastric secretion of 1.0 mg. of histamine injected intramuscularly was also determined in gastric fistula dogs to provide a basis for comparison.

A CONSEQUENCE of the widespread use of reserpine has been the appearance of numerous reports concerning the type and incidence of side reactions associated with extensive and prolonged administration of the drug. Among the untoward effects most frequently cited are nasal congestion, drowsiness, fatigue, excessive appetite, bizarre dreams, and gastrointestinal disturbances such as abdominal cramps and looseness of the bowels.

In many respects the pharmacologic actions of reserpine mimic those resulting from stimulation of the parasympathetic nervous system, although the mechanism has been explained on the basis of central inhibition of sympathetic activity (1, 2). Bein (3) and Plummer and coworkers (4) observed increased motor activity of the intestinal tract of intact animals following administration of reserpine which, according to others (5, 6), is generally associated with moderate to severe diarrhea. Plummer and coworkers (4) and Barrett and associates (7) reported that reserpine increased volume and hydrochloric acid content of the gastric juice of dogs, both in the vagally innervated gastric fistula pouch and in the vagally denervated Heidenhain pouch. This hypersecretion was found to be effectively prevented by subcutaneous administration of either an anticholinergic or a ganglionic blocking agent. It therefore appears that the gastric secretory effects of reserpine may be at least in part peripheral. Packman, *et al.*, (8) obtained comparable increases in gastric acid secretion in dogs with intravenous injections of 11-desmethoxyreserpine.

Kert, *et al.*, (9) reported one patient who

* Received March 14, 1957, from the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Philadelphia College of Pharmacy and Science.
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† Director, LaWall and Harrisson Laboratories, 1921 Walnut St. Philadelphia, Pa.

suffered an apparent exacerbation of peptic ulcer symptoms during a course of reserpine therapy. Beman, *et al.*, (10) found that both oral and intravenous preparations of reserpine produced significant elevation of gastric acidity in man, which was not repressed by administration of atropine. Similar results were obtained by Wolf and Rossman (11). On the other hand, Rider (12) reported that prolonged oral administration of reserpine did not elicit any consistent gastric secretory change in humans.

At this time the literature contains a rather complete documentation of both the therapeutically desirable and toxic effects of reserpine and chlorpromazine, the first ataraxics to achieve widespread acceptance. The more recently introduced tranquilizing agent, meprobamate (Miltown or Equanil), has also enjoyed enthusiastic reception by both the medical profession and the general public. Considering the extensive use of this chemical in a wide variety of clinical states in which anxiety, tension, and agressiveness play a prominent role, the appearance of reports related to the occurrence of adverse reactions was perhaps not unexpected. Undesirable reactions accompanying the use of meprobamate include drowsiness, skin rashes (chiefly purpuric in nature), muscular, gastrointestinal, and cerebral effects (13, 14, 15) including possible habit formation (16). Review of these reactions has led to the suggestion that meprobamate may belong to the class of compounds designated as histamine liberators (17). It is not suggested that histamine release is the mechanism of gastric acid secretion induced by meprobamate, as reported in this paper, but the relationship does appear worthy of further investigation.

The purpose of this study was to evaluate the secretory effects of oral and parenteral doses of reserpine, and oral administration of meprobamate in pyloric ligated rats, and to quantitate the gastric acid stimulating response to reserpine in gastric fistula dogs.

EXPERIMENTAL

The technique employed in the rat study was essentially that described by Shay (18) with minor modification. Female Sherman rats weighing 150 to 175 Gm. were fasted twenty-four to thirty hours but allowed free access to water. Ethyl ether was used as the anesthetic during pyloric ligation as pentobarbital was found to markedly depress gastric secretion. In all series the rats were sacrificed and the stomachs were removed four hours after ligation. Volume of gastric contents as well as free and total titratable acidity were measured. Free titratable acidity was determined using Töpfer's reagent (dimethyl-amino-azobenzene) as the indicator, while phenolphthalein was employed in assaying total acid. Results of these titrations are expressed in milliequivalents (meq.) free hydrochloric acid per ml. gastric sample.

Treatment of the experimental groups prior to ligation was as follows: Group A, control, distilled water 1.0 ml./Kg., *per os*, two hours before ligation (N = 43);[1] Group B, reserpine 0.5 mg./Kg., s.c., immediately after ligation (N = 18); Group C, oxyphenonium (Antrenyl®) 0.5 mg./Kg., s.c., five minutes before and reserpine 0.5 mg./Kg., s.c. immediately after ligation (N = 19); Group D, reserpine 0.5 mg./Kg., *per os*, once daily for four days, pyloric ligation eight hours after the last dose (N = 34); Group E, reserpine 0.5 mg./Kg., *per os*, four hours prior to ligation (N = 18); Group F, reserpine 0.5 mg./Kg., *per os*, two hours before ligation (N = 19); Group G, meprobamate 15 mg./Kg., *per os*, two hours before ligation (N = 18). In all experiments the calculated doses were dissolved or suspended in a constant volume (1.0 ml./Kg.) of distilled water, with the exception of reserpine for parenteral administration, which was dissolved in a constant volume of solvent consisting of a 1:1:2 ratio of propylene glycol, ethanol, and distilled water. An equivalent amount of solvent alone was found to have no effect on gastric secretion.

For the dog study, mongrel dogs of both sexes weighing 10 to 12 Kg. were prepared with a Pavlov simple gastric fistula. A polyethylene cannula, previously described (19), was inserted into the fistula to reduce contamination during gastric sampling. The dogs were fasted for twenty-four hours and injected intramuscularly with either 1 mg. of reserpine (N = 6), 20 mg. of meprobamate (N = 6), or 1 mg. of histamine (N = 6). Gastric samples were collected fifteen minutes after administration of the drug being tested and every fifteen minutes thereafter over a seventy-five-minute period. Free and total titratable acidity and pH of each sample were determined.

RESULTS AND DISCUSSION

In the regimen employed, 0.5 mg./Kg. of reserpine administered either orally or subcutaneously did not significantly increase the volume of gastric fluid secreted by the Shay rat during four hours after pyloric ligation. However, both free and total titratable acidity were appreciably increased following either oral or subcutaneous administration of reserpine. Unlike certain of the central nervous system manifestations of reserpine action, the gastric acid stimulating effect appears to be rapid in onset and of comparatively short duration. The secretory response to reserpine does not appear to depend upon cumulation of the drug resulting from administration over a number of days (Group D). A progressively greater increase in gastric acidity was obtained as the time interval from reserpine administration until pyloric ligation was shortened (Fig. 1.). Maximal stimulation was observed when reserpine was given only two hours before ligation (Group F). This time interval was not further reduced in order to avoid the possibility of incomplete gastric emptying and the presence of remaining drug in the stomach which might interfere with the

[1] The figures in parentheses represent the number of animals used in each experiment.

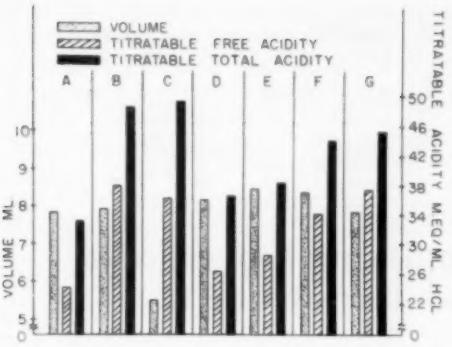

Fig. 1.—The effects of reserpine and meprobamate on the volume of gastric fluid secretion, free and total titratable acidity in the pyloric ligated rat. **A** = control; **B** = reserpine 0.5 mg./Kg., s.c., immediately after pyloric ligation; **C** = oxyphenonium 0.5 mg./Kg., s.c., 5 minutes before, and reserpine 0.5 mg./Kg., s.c., immediately after ligation; **D** = reserpine 0.5 mg./Kg., orally, once daily for 4 days with last dose 8 hours before pyloric ligation; **E** = reserpine 0.5 mg./Kg., orally, 4 hours before ligation; **F** = reserpine 0.5 mg./Kg., orally, 2 hours before ligation; **G** = meprobamate 15 mg./Kg., orally, 2 hours before ligation.

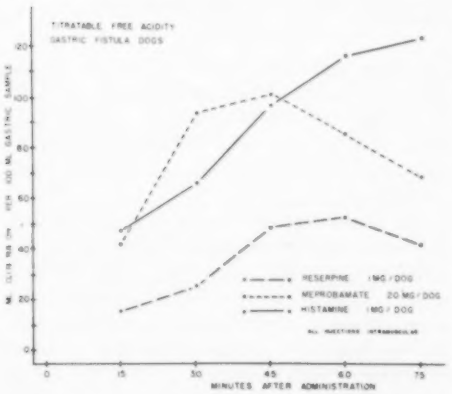

Fig. 2.—Comparison of the effects of intramuscular injection of reserpine, meprobamate and histamine on the titratable free acidity in gastric fistula dogs.

analyses. Subcutaneous injection of reserpine induced greater production of free and total titratable acidity than the same dose administered orally (Group B).

Experimental evidence has been provided by Barrett, *et al.*, (7) which indicates that reserpine evokes gastric secretion by stimulation of the parasympathetic ganglia. Clark and Schneider (20) found that the hyperchlorhydria induced by intravenous doses in humans was only partially suppressed by methantheline bromide (Banthine®) and was unaffected by atropine or epinephrine. According to our results, oxyphenonium, a cholinergic blocking agent, significantly reduced the volume of gastric fluid secreted in response to subcutaneous reserpine but did not influence free or total acidity per ml. of sample collected (Group C). Apparently the enhanced gastric secretion is not entirely dependent on a functional nerve supply to the gastric mucosa.

Oral administration of 15 mg./Kg. of meprobamate two hours before pyloric ligation did not alter the amount of gastric fluid secreted during the succeeding four-hour interval, but markedly increased both free and total titratable acidity. Friedman and Marmelzat (13) noted intestinal hyperactivity associated with meprobamate therapy, but no reports of meprobamate induced gastric hypersecretion have been observed in the literature.

Intramuscular injection of 1 mg. of reserpine consistently increased gastric acid secretion in dogs prepared with Pavlov simple gastric fistulas (Fig. 2). Titratable free acidity reached a peak about one hour after reserpine injection. Meprobamate injected intramuscularly also increased gastric acid secretion in the fistula dog. The response to 20 mg. of meprobamate was somewhat more pronounced but of shorter duration than that elicited by 1 mg. of reserpine. Neither compound evoked as great a gastric secretory response in the dog as 1 mg. of histamine.

SUMMARY

1. Reserpine, administered orally and subcutaneously, and meprobamate, given orally, did not appreciably alter the volume of gastric fluid secreted by pyloric ligated rats, but significantly increased both free and titratable acidity.

2. Reserpine and meprobamate, injected intramuscularly stimulated gastric secretion in dogs prepared with Pavlov simple gastric fistulas.

3. The gastric secretory response to reserpine in both the pyloric ligated rat and gastric fistula dog was rapid in onset and of relatively short duration in comparison to the tranquilizing and antihypertensive effects of the alkaloid.

4. Oxyphenonium, an anticholinergic agent, markedly reduced the volume of gastric acid secreted following parenteral administration of reserpine, but did not decrease titratable acidity of the gastric samples.

5. This study is currently being extended to other ataraxics presently employed in clinical practice.

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The Solubility and Complexing Properties of Oxytetracycline and Tetracycline I.*

Interaction in Aqueous Solution

By EUGENE H. GANS† and TAKERU HIGUCHI

Oxytetracycline dihydrate and tetracycline trihydrate form soluble complexes with sodium salicylate, sodium parahydroxy benzoate, sodium saccharin, sodium *p*-amino benzoate, caffeine, and N-methyl pyrrolidone in aqueous solution. In all cases the tetracycline interactions were greater than those with oxytetracycline. With the exception of sodium salicylate at concentrations below 0.5 *M*, the solubilizing activities of these compounds are roughly in the order listed above. Logarithmic plots show a linear relationship in dilute solution and the slope values of these curves reveal that an increase in solubility of the tetracyclines is concomitant with an increase in the complexing agent dependency. The solubilization may be due, in part, to the presence of strong electronegative centers within the agents used. Two recrystallization procedures for the preparation of oxytetracycline and tetracycline hydrates are described.

THE STRUCTURAL FORMULAS of oxytetracycline and tetracycline in Fig. 1 reveal the complexity of these amphoteric molecules. An insight into their chemical, physical, and solution behavior can be attained by reference to the works of Regna, *et al.* (1), and Hochstein and coworkers (2), Stephens, *et al.* (3), Boothe, *et al.* (4), and to the Merck Index (5), among others. Recent reports (6, 7) indicate that the dimethylamine group on C.4 undergoes a racemization to produce epimers of the individual tetracyclines. The tetracyclines form well defined salts with mineral acids and bases. Higuchi, Gupta, and Busse (8) determined that the logarithm of oxytetracycline solubility in aqueous acidic media is a linear function of pH, obeying the equation: $\text{Log}\ (S - S_0) = \text{Log}\ S_0 + p\text{Ka} - \text{pH}$. The high solubility of these salts is in marked contrast to the aqueous insolubility, i.e., *ca.* 10^{-4} mole/L., of stable oxytetracycline dihydrate and tetracycline trihydrate at the isoelectric points. The comparatively low solubility may well be attributed to formation of inter- and intramolecular hydrogen bonding by the various protophilic and protodotic groups on the molecule.

The present communication is concerned with the complexing tendencies in water of the two antibiotics with salts of organic acids and with certain neutral nitrogenous compounds. Because of their polar and polyfunctional characters, the two drugs may well be expected to associate molecularly with suitable complexing agents thus modifying their solubility and absorption characteristics.

Previous workers have mainly confined their studies to the effect of metal ions upon the tetracyclines. Regna and co-workers (1) detected calcium chloride complexes of oxytetracycline by the resulting shift in *p*Ka values upon titration with base. Using the same technique, Albert (9) described metal complexes with ferrous, ferric, cuprous, nickel, cobaltous, zinc, and magnesium chlorides. In two excellent papers, Ishidate and Sakaguchi (10), and Sakaguchi and Taguchi (11), indicated that the *peri* hydroxyl and ketone functions of the enolized 1,3-β-dicarbonyl system in chlortetracycline (C.11—C.11a—C.12) undergo

* Received January 25, 1957, from the School of Pharmacy, University of Wisconsin, Madison.
Presented before the Scientific Section, A. PH. A., New York meeting, April, 1957.
This paper is based in part on a thesis submitted to the Graduate School of the University of Wisconsin by Eugene H. Gans in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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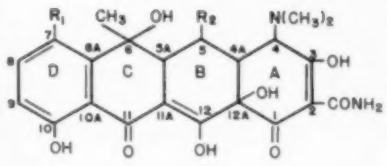


COMPOUND	R_1	R_2
CHLORTETRACYCLINE	Cl	H
OXYTETRACYCLINE	H	OH
TETRACYCLINE	H	H

Figure 1.

chelation reactions with thorium, zirconium, uranium, aluminum, magnesium, etc., of a 1:1 or a 1:2 type. The 1:1 chlortetracycline-thorium chelate was utilized as a basis for a sensitive method of analysis of the antibiotic. The authors infer that oxytetracycline and tetracycline should also undergo analogous chelation reactions.

THEORETICAL CONSIDERATIONS

The terminology applied to the solubilization of an insoluble solute by the addition of a third component is indeed variable. The increase in solubility due to the addition of an inorganic salt is known as "salting in," or if the reverse occurs, as "salting out"; the Debye-Huckel law or its extensions being capable of describing the simpler cases. If the solubilizing additive does not interact appreciably with the mutual solvent, the system can be called an ideal case of mixed liquid solvents; and if competitive reactions occur, the system is then nonideal. When micelle formation, by the third component, is suspected or known, then the phenomenon is termed "true solubilization" by McBain (12). A mixture of two solvents, each one usually a poor solvent for the particular solute, to produce a solvent of high activity has been called "co-solvency." In 1916, Neuberg (13) introduced the term "hydrotropy" to account for the solubility increase due to the addition of aromatic carboxylic or sulfonic acid salts. Neuberg and Roberts (14) showed that aromatic sodium benzoate was more effective than alicyclic sodium hexahydroxybenzoate, and von Hahn (15) claimed that acetone, pyridine, alcohol, and acetic acid also displayed hydrotropic action. The typical hydrotropic solubility curve is not a linear function of the concentration of the hydrotropic agent, but has considerable sigmoidal character. Booth and Everson (16–18) and Licht and Weiner (19) have studied the hydrotropic action of various benzene, toluene and xylene sulfonates. The latter concluded that the hydrotropic action increases with an increase in the number and size of the alkyl groups on the benzene nucleus, and also with an increase in temperature.

With the exception of definite micelle formation or cases where the Debye–Huckel law is applicable, reasonable theoretical explanations of these individual phenomena have been generally lacking. Whereby solubilization terminology is generally used so that individual systems are classified according to the particular agent utilized, new situations arise which either do not fit in with the present scheme or are apparent overlapping examples of two or more systems.

In the present study, where the systems are nonideal for the most part, and where the strong polarity of the tetracyclines, water, and solubilizing agents suggest the presence of simultaneous competing interactions, the terminology of complex formation is preferred. Although complex formation is broad in scope, its basis is the nature and deployment of the various electrostatic forces involved in the system; thus allowing formulation of considerations based on the known chemical and physical characteristics of the interacting components.

Higuchi and Lach (20), working with soluble xanthine complexes, suggested that soluble complex formation can be due to a replacement reaction, whereby the acidic hydrogens of the solute coordinate with the negative centers of the complexing agent to replace water from these negative groupings. Furthermore, the displacement reaction may be rendered more favorable by the "squeezing out" action of water molecules upon the hydrophobic, lipophilic portions of the interacting molecules; thereby allowing the lipophilic portions of the solute and complexing agent to interact to a greater extent than would be expected from dipole-dipole considerations. In dealing with compounds having aromatic character, the effect of π complex formation may have a synergistic action. In the case of the tetracyclines, the strong solvates formed with toluene are difficult to explain except on a basis of unusually strong π complex formation.

Since many hydrotropic salts, e.g., sodium benzoate, salicylate, toluenesulfonate, xylenesulfonate, cymenesulfonate, etc., as well as the agents used herein, possess strong negative groupings, it appears logical that the displacement reaction cited above may play an important role in the solubilization processes. The tetracyclines offer three acidic hydrogens, two enolic at C.3 and C.11 and one phenolic at C.10, and other less active hydrogens for interaction with protophilic groupings. The partially aromatic nature of the tetracycline nucleus may induce increased lipophilic interaction, possibly through formation of π complexes.

The tetracyclines probably exist as zwitter ions in solution, in which case the enolic hydrogen at C.3 would be attached to the dimethyl amino group on C.4 and the zwitter ion would be composed of the dimethylammonium ion and various resonance forms of the negatively charged beta-dicarbonyl system (C.1–C.2–C.3) on ring A.

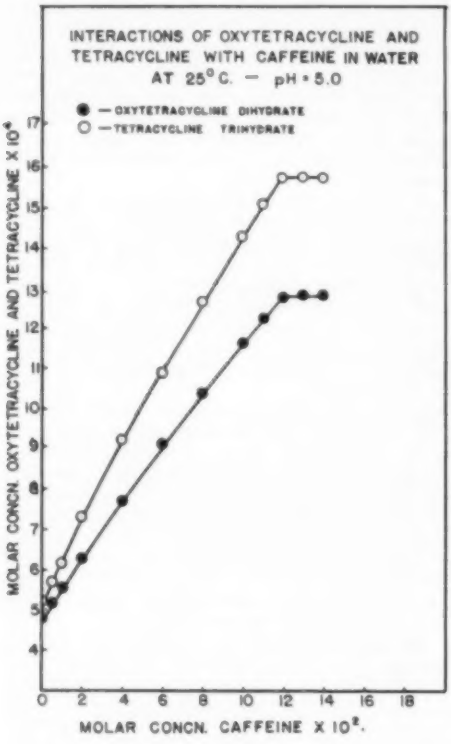

INTERACTIONS OF OXYTETRACYCLINE AND TETRACYCLINE WITH CAFFEINE IN WATER AT 25°C. — pH = 5.0
● — OXYTETRACYCLINE DIHYDRATE
○ — TETRACYCLINE TRIHYDRATE
MOLAR CONCN. OXYTETRACYCLINE AND TETRACYCLINE X 10⁴
MOLAR CONCN. CAFFEINE X 10².

Figure 2.

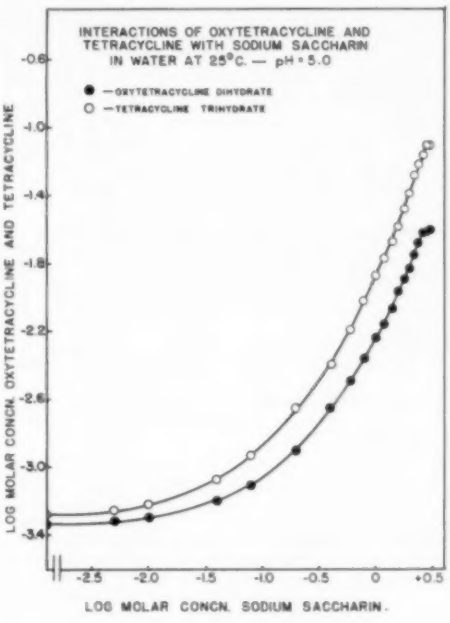

INTERACTIONS OF OXYTETRACYCLINE AND TETRACYCLINE WITH SODIUM SACCHARIN IN WATER AT 25°C. — pH = 5.0
● — OXYTETRACYCLINE DIHYDRATE
○ — TETRACYCLINE TRIHYDRATE
LOG MOLAR CONCN. OXYTETRACYCLINE AND TETRACYCLINE
LOG MOLAR CONCN. SODIUM SACCHARIN.

Figure 3.

EXPERIMENTAL PROCEDURE

The formation of interaction species was detected by the method of solubility analysis. Ten cubic centimeters of an aqueous solution of known concentration of the particular complexing agent was equilibrated with an excess of either oxytetracycline dihydrate or tetracycline trihydrate at 25° ± 0.1°, in the constant temperature bath described by Higuchi, Gupta, and Busse (8). Clear glass shell vials, of 15 cc. total capacity and stoppered by pure gum rubber closures, were used as interaction vessels. The solutions were maintained at constant pH by suitable buffers, acetate buffer at pH 5.0 and phosphate buffer at pH 6.4. Sodium bisulfite, 0.1%, was employed as an antioxidant and the general equilibration time of twelve hours was chosen so as to allow the solutions to approach maximum equilibration and yet keep degradative processes at a minimum.

After equilibration, the excess antibiotic was allowed to settle, the liquid filtered through a glass wool plug into a volumetric pipette, and the resulting clear solution of the particular tetracycline was suitably diluted for spectrophotometric measurement (*vide infra*).

RESULTS AND DISCUSSION

Caffeine.—Previous workers in these laboratories have shown that caffeine forms both soluble and insoluble complexes with a large number of organic compounds. Its successful use in this prior work suggested the employment of caffeine herein. As shown in Fig. 2, the solubilities of both oxytetracycline and tetracycline are increased approximately threefold. The relationship, as depicted, is not linear as the slope decreases gradually with increasing caffeine concentration. The curves end abruptly at a caffeine concentration of 1.2×10^{-1} moles per liter, which is the solubility limit of caffeine in these solutions. It was interesting to note that the solubility of caffeine at 25° and at pH 5.0, in the absence of the tetracyclines, was 1.1×10^{-1} moles per liter. In other words, interaction with the tetracyclines caused an increase in caffeine solubility of 1×10^{-2} moles per liter at this temperature. Calculations using this figure as the amount of caffeine interacting with the solubilized tetracyclines yield rather high and uncertain stoichiometric ratios. Exact determination of the stoichiometry is made difficult because of the existence of the caffeine monomer-dimer-tetramer equilibrium recently elucidated by Guttman and Higuchi for pure caffeine in water (21).

Sodium Saccharin.—The free acidic imide, saccharin, is a rather strong acid; Ka is *ca.* 1.3×10^{-2}, comparable to salicylic acid. Thus the saccharate ion is quite strongly negative and should have considerable attraction for the acidic hydrogens. The large increase in the solubility of the tetracyclines, noted in Fig. 3, appears to substantiate this view. The interaction has been plotted on a log-log scale for the sake of graphical convenience. Thus, upon the addition of a 2.8 *M* solution of sodium saccharin at pH 5.0 to oxytetracycline dihydrate, the solubility of the latter increased from 4.54 to 10^{-4} *M*

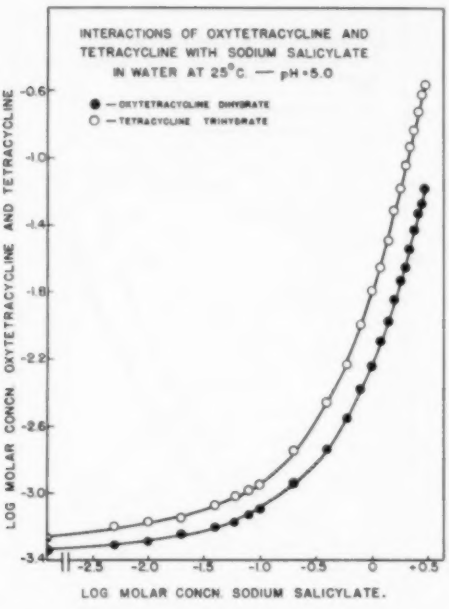


Figure 4.

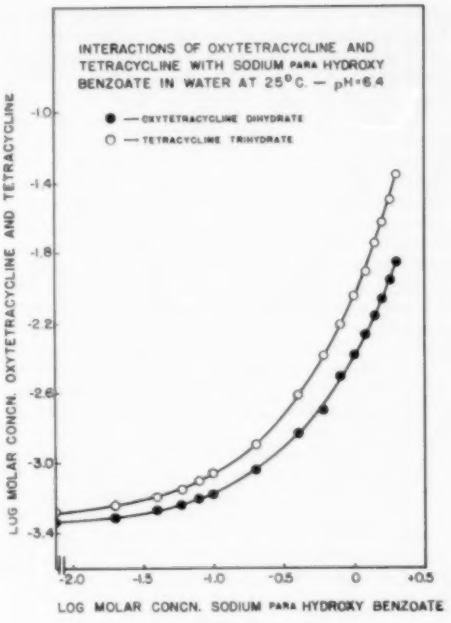


Figure 5.

per liter to 2.50×10^{-3} *M* per liter, an approximately 55-fold increase. Tetracycline trihydrate, under identical conditions, was solubilized roughly 150 fold, from 5.25×10^{-4} *M* per liter to 7.95×10^{-2} *M* per liter at 25°. The shapes of the solubility curves for the saccharin interaction, and for the succeeding interactions, are rather typical of hydrotropic solubility curves reported in the literature. The solubility limit of sodium saccharin was reached at a concentration of 2.8 *M*, and addition of excess above this point produced no change in the maximum solubility of the tetracyclines in this system.

In order to test the effect of comparable concentrations of inorganic salts, runs were made using sodium chloride and sodium nitrate. At 2 *M* concentrations, the effect of the former was negligible, while there was a small solubility increase using sodium nitrate. Thus, the solubilizing effect appears to be due to the anion characteristics, and in these latter interactions the oxygens of the nitrate ion apparently coordinate to a higher degree than the chloride ion.

Sodium Salicylate.—Sodium salicylate is the salt of the strongest acid used in these studies, Ka of the free acid being 1.06×10^{-3} at 25°. The results, presented in Fig. 4, show that the solubilization of the tetracyclines at salicylate ion concentrations of 2.8 and 3.0 *M* is greater than that produced by sodium saccharin. Oxytetracycline dihydrate solubility in a 3.0 *M* solution of the salt at 25° was 6.58×10^{-2} *M* per liter, an increase of 145 fold. At the same concentration of salicylate ion, tetracycline trihydrate was soluble to the extent of 2.73×10^{-1} moles per liter, this being 525 times the initial solubility. At concentrations of 2.8 molar, which was the limit of effective sodium saccharin activity, the increases are 118 fold and 450 fold, respectively.

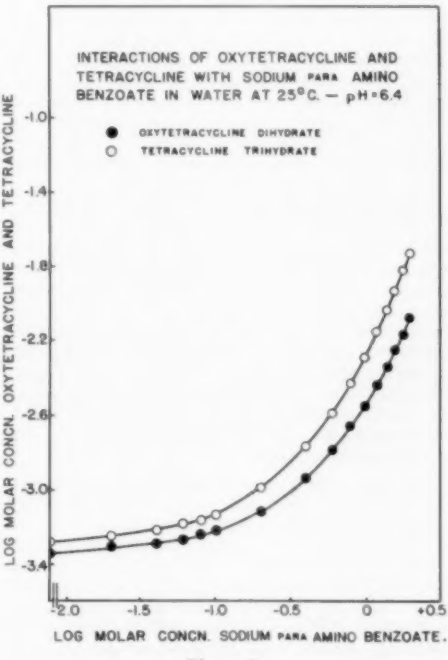


Figure 6.

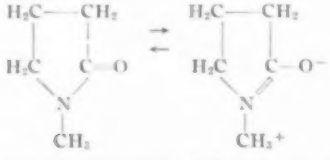
Sodium Para-Hydroxybenzoate.—The interactions of oxytetracycline dihydrate and tetracycline

trihydrate with the sodium salt of para-hydroxy benzoic acid are depicted in Fig. 5. The acid has a pKa of 4.54 at 25°, limiting the maximum working concentration at 2.0 molar, the equilibrium solubility at pH of 6.4. While the effect of moving the hydroxyl group to the para position of the benzene nucleus caused a considerable reduction in the pKa value, as compared to salicylic acid, the solubilization of the tetracyclines, however, was not reduced proportionately. At the maximum concentration of 2.0 molar of sodium para-hydroxy benzoate, the solubilities of the antibiotics are almost identical with those attained when sodium saccharin was employed at the same concentration and are approximately one half the solubilities induced by an equal concentration of sodium salicylate. The equilibrium solubility of oxytetracycline dihydrate in presence of 2.0 molar sodium para-hydroxy benzoate was 1.41×10^{-2} moles per liter, while that of tetracycline trihydrate was 4.38×10^{-2} moles per liter.

Sodium Para-Aminobenzoate.—The amino analog of para-hydroxy benzoate, pKa of the free acid is 4.92 at 25°, induced the smallest increase in solubility of the four salts used herein. The results, presented in Fig. 6, show the equilibrium solubility of oxytetracycline dihydrate at 2.0 molar concentration of the para-amino salt to be 8.31×10^{-3} moles per liter, while that of tetracycline trihydrate is 1.87×10^{-2} moles per liter. The solubility increases are 18 fold and 35 fold respectively.

It will be noted that here, as in the other systems, the complexing tendency of tetracycline is greater than that of oxytetracycline. Since the two antibiotics differ only by the C.5 hydroxyl group of the latter, reasons for the variance in interaction affinities between the two are not readily apparent. The C.5 hydroxyl could play an important role sterically and/or could be involved in the formation of inter- or intramolecular hydrogen bonds between one or more oxytetracycline molecules.

N-Methyl Pyrrolidone.—N-methyl pyrrolidone[1] is a lactam, specifically γ-N-methyl butyrolactam, and has been recently introduced in bulk quantities as a commercial solvent. It is completely miscible with water and its solution is relatively neutral. In the pure state, it evidently possesses, because of its unexpectedly high boiling point, an organized structure. Since the addition of water causes heat to be evolved, the lactam appears to have considerable affinity for water molecules. In solution, one might consider it to be composed of several resonance forms, the two extremes of which could be represented as follows:



The interactions were studied up to a concentration of 5.0 M, after which point the lactam assumed the major portion of the solvent volume and the measured pH of the system would then only be the apparent pH. The results, presented in Fig. 7, show the solubilization of the two tetracyclines proceeding rather slowly in comparison to our other systems. Only at relatively high concentrations of the lactam do the equilibrium solubilities of the antibiotics increase more rapidly. At 5.0 M N-methyl pyrrolidone, oxytetracycline dihydrate solubility is 4.62×10^{-2} moles per liter, while that of tetracycline trihydrate is 1.27×10^{-2} moles per liter at 25°. The solubility of the two antibiotics *in* one liter of pure N-methyl pyrrolidone at 25° is rather interesting:

Tetracycline trihydrate—2.17 moles
Oxytetracycline dihydrate—0.70 mole

Not only is the tetracycline approximately three times as soluble in the pure lactam as is oxytetracycline, but the solution time of the former is quite rapid as compared to that of the latter. When tetracycline trihydrate is dissolved in pure N-methyl pyrrolidone and water is then added slowly, highly crystalline hydrated tetracycline base deposits after a short period of standing. This was used as the basis for a new recrystallization method described later in this paper.

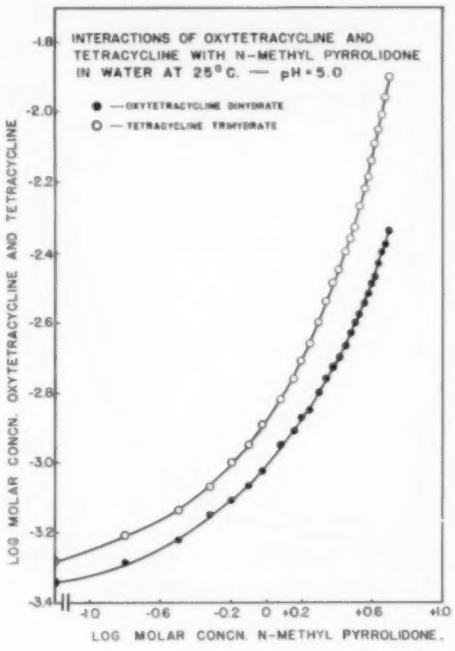


Figure 7.

Complexing Agent Dependencies.—When the log molar concentrations of oxytetracycline dihydrate or tetracycline trihydrate solubilized were plotted against the log molar concentrations of complexing agent, for all systems except caffeine, a linear relationship was noted over a considerable range of the more dilute solutions. Thus, the logarithm of antibiotic solubility, at complexing agent concentration, X, less the solubility of the

[1] We are indebted to General Aniline and Film Corp., New York, N. Y., commercial producers of N-Methyl Pyrrolidone, for providing us with a generous sample.

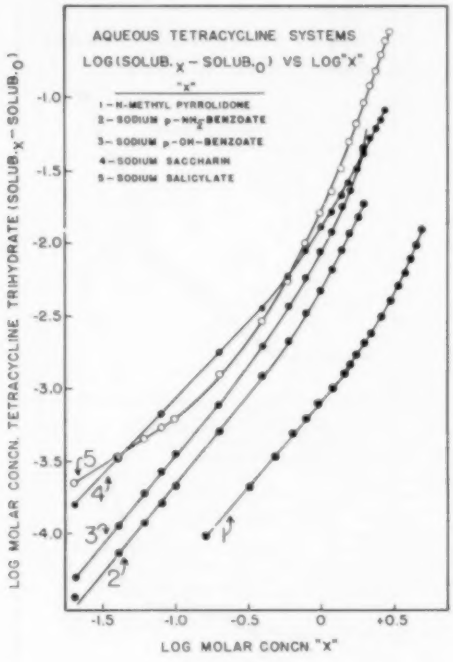


Figure 8.

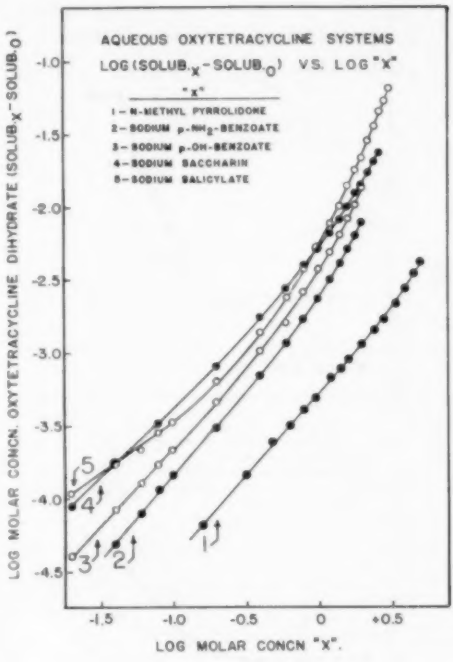


Figure 9.

antibiotic in water, has been plotted against log molar concentration of complexing agent (i.e. log S_x − log S_0 *vs.* log X). The plots representing aqueous tetracycline systems are shown in Fig. 8, and those for aqueous oxytetracycline systems in Fig. 9. The diagrams representing both systems have several things in common. First, it may be noted that the linear slopes for all interactions, except those of sodium salicylate, are approximately the same. Secondly, the placement of an individual curve on the graph, with respect to the neighboring curves, is indicative of the magnitude of the interaction. It will be noted, next, that the sodium salicylate curves, no. 5 in each figure, have shorter linear portions of smaller slope initially, followed by relatively steep curvatures resulting in short linear portions of high slope value at higher salt concentrations. The curves representing sodium para-hydroxy benzoate, no. 3 in each figure, are regular at the beginning, and then also begin to curve steeply, although they are not linear at high salt concentrations. The same effect, although of decreased magnitude, is displayed by curve no. 1, representing the N-methyl pyrrolidone interactions.

The linear portions of the curves can be described by the exponential equation of the type: $y = bx^a$, where both a and b are constants. The constant, b, is undoubtedly composed of several factors pertaining to the magnitude of several simultaneous interactions taking place.

The constant, a, or the slope value, when plotted against log X, indicates the tetracycline or oxytetracycline-complexing agent dependency. In Fig. 10, for aqueous tetracycline systems, and in Fig. 11, for aqueous oxytetracycline systems, the slope values, i.e. d log complex/d log X at representative points on each curve in Figs. 8 and 9, have been plotted against the logarithm of the concentration of the solubilizing agent. As seen in Fig. 10 for tetracycline systems, the saccharin interaction is initially first order with respect to the salt while the others, except for sodium salicylate, appear to be slightly higher than first order. The salicylate interaction initially has a dependency value on the other hand of ca. 0.6. As the concentration of salicylate ion increases the dependency appears to increase progressively. At higher concentrations of the other agents, the saccharin curve appears to be approaching a limiting value of two, while the para-amino benzoate, the para-hydroxy benzoate, and the N-methyl pyrrolidone curves are seen to be rising sharply with increasing concentrations.

In Fig. 11, representing oxytetracycline systems, the considerations for the para-amino benzoate, para-hydroxy benzoate, and N-methyl pyrrolidone curves are about the same as in Fig. 10, although the steep rises are not as pronounced. The salicylate curve here is approximately similar to that for tetracycline. The oxytetracycline–saccharin curve is markedly different from its counterpart shown in Fig. 10, the former being very similar, except at high concentrations, to that representing the salicylate-tetracycline interaction.

The dependency values described above should only be considered as indications of what might be occurring in solution, since isolation of the complexes was not possible and the use of more accurate

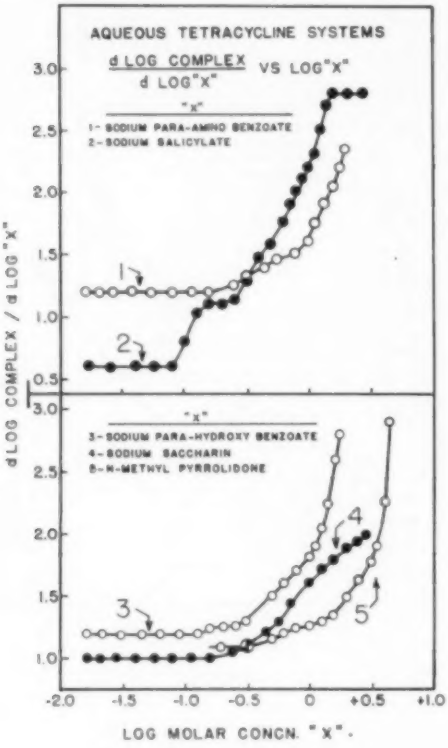

AQUEOUS TETRACYCLINE SYSTEMS
d LOG COMPLEX / d LOG "X" VS LOG "X"
"X"
1- SODIUM PARA-AMINO BENZOATE
2- SODIUM SALICYLATE
3- SODIUM PARA-HYDROXY BENZOATE
4- SODIUM SACCHARIN
5- N-METHYL PYRROLIDONE
d LOG COMPLEX / d LOG "X"
LOG MOLAR CONCN. "X".

Figure 10.

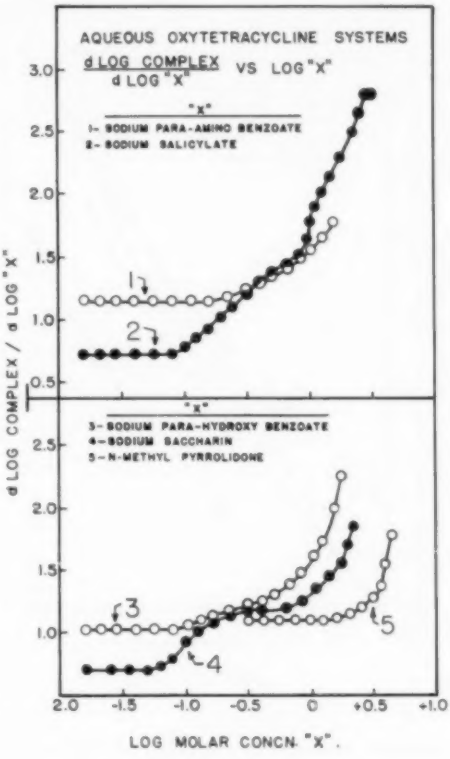

AQUEOUS OXYTETRACYCLINE SYSTEMS
d LOG COMPLEX / d LOG "X" VS LOG "X"
"X"
1- SODIUM PARA-AMINO BENZOATE
2- SODIUM SALICYLATE
3- SODIUM PARA-HYDROXY BENZOATE
4- SODIUM SACCHARIN
5- N-METHYL PYRROLIDONE
d LOG COMPLEX / d LOG "X"
LOG MOLAR CONCN. "X".

Figure 11.

methods of stoichiometric analysis involved serious difficulties.

EXPERIMENTAL

Reagents.—*Caffeine:* Recrystallized caffeine U. S. P., m.p. 235–237°.

Sodium saccharin: Recrystallized sodium saccharin, U. S. P., dried for eight hours at 110°.

Sodium salicylate: Recrystallized sodium salicylate, U. S. P., dried for eight hours at 110°.

Sodium p-hydroxy benzoate: Prepared by neutralization, with sodium carbonate, of recrystallized para-hydroxy benzoic acid. The resulting salt was recrystallized from water-methanol and dried *in vacuo* at 50° for eight hours.

Sodium p-amino benzoate: Recrystallized from sodium *p*-amino benzoate, Matheson-7396. Dried *in vacuo* at 50° for eight hours.

N-Methyl pyrrolidone: General Aniline and Film Corp., Batch 503. Mol. wt. 99.4, b.p. 202°. Redistilled at 95° and 22 mm., f.p.—24.8°.

Sodium bisulfite: Sodium bisulfite, reagent grade.

Sodium acetate: Sodium acetate, analytical grade.

Acetic acid: Glacial acetic acid, analytical grade.

Sodium acid phosphate: Sodium acid phosphate, analytical grade.

Disodium phosphate: Disodium phosphate, analytical grade.

Preparation of Oxytetracycline Dihydrate and Tetracycline Trihydrate.—The starting materials were either relatively pure crystalline hydrochloride salts or less pure samples of the bases. Two methods of recrystallization were evolved to produce crystalline hydrated bases. The first, method A, involved neutralization of a solution of the hydrochloride salt or an acid solution of the base with sodium acetate. The second, method B, was applicable only to tetracycline base and involved recrystallization of the latter from N-methyl pyrrolidone. As a general rule, oxytetracycline dihydrate was prepared solely by method A and tetracycline trihydrate by use of both methods or procedure B alone, depending upon the starting material.

The stable hydrates were produced by drying, *in vacuo*, at room temperature for twelve hours. The water content of the prepared batches were checked by Karl Fischer analysis and by nonaqueous titration of the bases in acetic acid, using perchloric acid in dioxane as the titrant and quinaldine red as the indicator. As a final check on purity and reproducibility, the solubility of the base hydrates were determined before use. The antibiotics were stored in desiccators until ready for use.

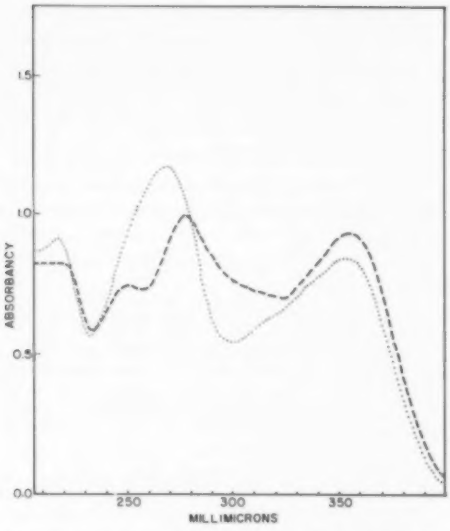


Figure 12.—Ultraviolet spectra of oxytetracycline: ..... dihydrate in HCl (0.1*N*), 3.00 mg. per 100 ml.—— dihydrate in *p*H 5.0 acetate buffer, 3.01 mg. per 100 ml.

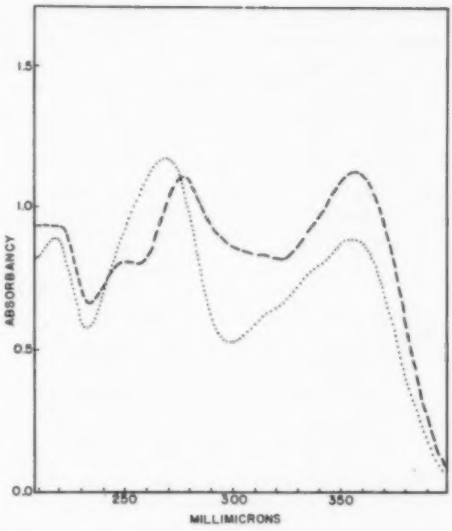


Figure 13.—Ultraviolet spectra of tetracycline: ..... trihydrate in HCl (0.1 *N*), 3.00 mg. per 100 ml. —— trihydrate in *p*H 5.0 acetate buffer. 3.54 mg. per 100 ml.

Method A.—Either the hydrochloride salt or the free base is dissolved in water by adding sufficient hydrochloric acid to bring the pH down to approximately 1.5. The low pH is necessary to prevent hydrolysis of the protonated base to the insoluble free base. The solution is filtered through suitable paper and sufficient sodium acetate, previously dissolved in water, is added slowly with stirring to bring the pH up to 5.0. The precipitated crystalline base is transferred to a Büchner funnel, washed several times with cold distilled water, and sucked dry. The cake is transferred to a suitable flask and slurried with about five times its volume of water to remove traces of salts or acetic acid from the crystals. The crystalline base is then dried at the pump, in a Büchner funnel, after washing once with water. The cake is broken up and dried, *in vacuo*, at room temperature over magnesium perchlorate for twelve hours. Where necessary, the process can be repeated several times before the final vacuum drying.

Method B.—The free base is dissolved in three times its weight of N-methyl pyrrolidone. To facilitate the dissolution, the base hydrate should be dry. The resulting solution is filtered through a sintered glass funnel of medium porosity and an equal volume of water is added rapidly with stirring. The temperature rises slightly, and after standing a few moments the solution begins to deposit crystals of the base. The mixture is allowed to stand several hours, away from light and with intermittent stirring. After transferring the crystals to a Büchner funnel and washing with water, they are slurried with 10% alcohol, transferred back to the Büchner funnel, washed with a small amount of the dilute alcohol, and dried at the pump. The crystals were then dried, *in vacuo*, at room temperature over magnesium perchlorate for twelve hours. The entire process, including the vacuum drying, was repeated until the solution of the base in N-methyl pyrrolidone was a light straw-yellow in color. This method was applicable only to tetracycline and the yields were on the order of 75%. No effort was made to recover the less pure second crop of tetracycline crystals from the mother liquor.

ANALYTICAL PROCEDURE

The analysis of tetracycline and oxytetracycline was performed spectrophotometrically. In all cases, except in the experiments employing sodium salicylate, the appropriately diluted solutions were measured in the pH range of 5–6, since the spectra of the complexing agents did not interfere with the absorption of the antibiotics in the dilutions used. The spectra of sodium salicylate, at higher concentrations of the salts, did interfere and measurements were made in 0.1 *N* HCl to remove the sodium salt from solution.

The ultraviolet spectra of oxytetracycline dihydrate in pH 5.0 acetate buffer and in 0.1 N HCl are presented in Fig. 12. The spectra of tetracycline trihydrate in the same media are presented in Fig. 13. Measurements were made using the maxima at 353 mμ for oxytetracycline and at 355 mμ for tetracycline. All measurements were made using either the Beckman model DU spectrophotometer or the Cary model XI recording spectrophotometer.

SUMMARY

1. Oxytetracycline dihydrate and tetracycline trihydrate form soluble complexes in aqueous

solution with a number of agents containing strong negative centers.

2. The degree of interaction, limited in certain cases by pH and solubility considerations, is roughly in the following order: sodium salicylate, sodium *p*-hydroxybenzoate, sodium saccharin, sodium *p*-aminobenzoate, caffeine, and N-methyl pyrrolidone. In all cases the tetracycline interactions were greater than those of oxytetracycline.

3. In dilute solution, the interactions show the following linear relationship: Log complex = log b + a log X, and in more concentrated solutions an increase in oxytetracycline or tetracycline solubility is concomitant with an increase in the complexing agent dependency.

4. Two recrystallization procedures for the preparation of the base hydrates have been described.

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Isolation of a Saponin, Hederin, and Its Sapogenin, Hederagenin, from Bur Clover (*Medicago hispida*)*

By E. D. WALTER†

A saponin isolated from the alcohol extract of fresh bur clover (*Medicago hispida*) has properties and constants identical with those of hederin. This saponin caused a marked increase in tonus and decreased peristalsis when in contact with isolated strips of rabbit ileum. The acidic sapogenin obtained by hydrolysis of the saponin was shown to be hederagenin $C_{30}H_{48}O_4$.

IN THE study of saponins in legume forages as a possible cause of bloat in ruminants, bur clover (*Medicago hispida*) was found to contain a saponin which was obtained in crystalline form. The saponin showed properties and constants identical with those of hederin, which was first isolated from ivy leaves (*Hedera helix*) by van der Haar (1). The sapogenin, hederagenin, has been isolated from various plants. Jacobs (2) obtained it by hydrolysis of the crude saponin of soapnuts (*Sapindus saponaria*). More recently hederagenin was isolated from the hydrolyzate of crude saponin from the seeds of *Nigella sativa* by Mustafa and Solima (3) and McShefferty and Stenlake (4) obtained it from *Caulophyllum thobitroides* (Berberidaceae) and from *Clematis vitalba* (Ranunculaceae). Thus a rather wide occurrence of this saponin is indicated.

Hydrolysis of bur clover saponin with 1 *N* hydrochloric acid in 70% alcohol gave a crystalline sapogenin (m. p. 332 to 333°), arabinose, and rhamnose. Analysis indicated that the sapogenin has the formula $C_{30}H_{48}O_4$ and that it contains a free carboxyl group as shown by titration with standard alkali. The presence of two hydroxyl groups was indicated by the formation of a diacetate, m. p. 170°. Further, the sapogenin was converted into its methyl ester, and this in turn into its acetonyl derivative. The constants of the sapogenin and derivatives are in agreement with those of hederagenin. Earlier workers (1, 2) gave the formula

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† The author is indebted to Arthur Bevenue for paper chromatography of the sugars, to Lawrence White and Geraldine Secor for elemental analyses, to Robert H. Wilson for physiological tests, and to Edith Gong for the infrared spectra.

$C_{31}H_{50}O_4$ for hederagenin but Kitasato (5) from extensive work on its structure proved it to be $C_{30}H_{48}O_4$.

Only one sapogenin, hederagenin, was isolated from the crystalline saponin and from the hydrolyzate of the crude extract of bur clover. This is in contrast to alfalfa (6) and ladino clover (7) each of which yielded several sapogenins.

A crystalline flavonoid, as yet unidentified, was also isolated from the alcoholic extract. Further work on this is in progress.

EXPERIMENTAL

Fresh bur clover (65 Kg.) was placed in a stainless-steel tank and covered with 217 liters of 95% alcohol, and allowed to stand for five days. The extract was drained, filtered, and concentrated to about one-third volume. It was then further concentrated on the steam bath, in four batches, until solids began to separate. Each batch was transferred to a separatory funnel and was shaken gently with about one-third volume of ether. After the mixture stood for several hours crystals appeared mainly at the interface between the aqueous and ether layers. Since some crystals floated in both layers, both were filtered and the crystalline material was washed with ether to remove green material and with water to remove carbohydrate material. The yield of saponin recrystallized from alcohol was 10 Gm., or about 0.07% of the dry weight of clover. The material softened on the Kofler melting point block (all melting points were taken on the Kofler block) at 225° and was fluid with browning at 256° $[\alpha]_D^{25}$ + 10° (in abs. alcohol, c, 0.8); van der Haar (1) + 9.68°.

Hydrolysis of crystalline saponin (0.5 Gm.) in 70% alcohol made 1 *N* with HCl yielded 0.3 Gm. of glistening rhombic prisms (see van der Haar (1) for a drawing and description of the crystallography of hederagenin), m. p. 332 to 333°, and arabinose and rhamnose in approximately equal amounts, as estimated and identified by paper chromatograms (van der Haar obtained 65.8% hederagenin, 16.8% arabinose, and 21% rhamnose on hydrolysis of hederin.)

Isolated strips of rabbit ileum were suspended in the usual manner in oxygenated Tyrode's solution, and contractions were recorded on a moving kymograph. Addition of 1 mg. of the saponin to 60 ml. of Tyrode's solution caused a marked increase in tonus and a decrease to complete disappearance of peristalsis and death of the tissues.

Hederagenin from Crude Concentrates.—Since not all the saponin would crystallize in the crude concentrates it seemed feasible to obtain the sapogenin by hydrolysis of the crude material. After removal of the crystalline saponin, the aqueous solution was made 1 *N* with hydrochloric acid and boiled for about 15 minutes. On cooling, the mixture set as a gel. The free liquid was filtered off, and the gel was dissolved in approximately 70% alcohol and refluxed for about 10 hours in approximately 1 *N* hydrochloric acid. After evaporation of some of the alcohol, water was added to precipitate the sapogenin. It was partially extracted with ether, but some was trapped in solids. The solids were dried and extracted with ether in a soxhlet. The combined ether extracts were clarified with charcoal, and the sapogenin was crystallized on evaporation of the ether. When crystallized from methanol and dried at 120° in a vacuum it melted at 332 to 333°, $[\alpha]_D^{25}$ + 77° ± 3° (in CH_2Cl-MeOH 1:1, c, 0.1), yield 18 Gm. from 14.4 Kg. dry weight of bur clover. Based on the observation that about 60% of the saponin is sapogenin, this would represent 30 Gm. of saponin. This amount plus the crystallized saponin (10 Gm.) would indicate that at least 0.27% of the dry weight of bur clover was saponin.

Anal.—Calcd. for hederagenin $C_{29}H_{47}O_2(CO_2H)$; C, 76.2; H, 10.2; neut. equiv. 472.4. Found: C, 75.9; H, 10.0; neut. equiv. 476 (titration with 0.05 *N* NaOH).

Hederagenin Diacetate.—Acetylation of sapogenin, m. p. 332°, by refluxing with acetic anhydride for 2 hours followed by recrystallization from 70% methanol furnished the diacetate, which sintered at 156° and became fluid at 170° (this behavior was observed by Jacobs (2) and McShefferty and Stenlake (4)), $[\alpha]_D^{25}$ + 64.2° (in abs. alc., c, 0.4). For analysis it was dried in a vacuum at 101°.

Anal.—Calcd. for $C_{29}H_{49}O_2(CH_3CO)_2(CO_2H)$: C, 73.3; H, 9.4; CH_3CO, 15.4. Found: C, 73.4; H, 9.37; CH_3CO, 16.2 (by saponification with alkali and back titration).

Hederagenin Methyl Ester.—The sapogenin (hederagenin) was esterified with diazomethane in ether solution. When recrystallized from methanol and dried in a vacuum at 110°, m. p. 238–240°; $[\alpha]_D^{25}$ + 71.1° (in $CHCl_3$, c, 0.4). The m. p. of a known sample of hederagenin methyl ester supplied by Dr. C. R. Noller of Stanford University was not depressed by mixture with this preparation.

Anal.—Calcd. for $C_{31}H_{50}O_4$: C, 76.5; H, 10.4. Found: C, 76.4; H, 10.3.

The infrared spectra of this material and that of authentic material were identical.

Acetonyl Hederagenin Methyl Ester.—The methyl ester (50 mg.) was dissolved in 5 ml. of dry acetone. When a few drops of concentrated hydrochloric acid was added, glistening leaflets at once appeared. Recrystallized from absolute alcohol, the compound formed platelets, m. p. 250°. This characteristic of hederagenin methyl ester was reported by Jacobs (2, p. 631) and by McShefferty and Stenlake (4). The m. p. of this material was not depressed when mixed with authentic acetonyl hederagenin methyl ester. The infrared spectra of this derivative and of that from authentic material were identical.

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Studies to Determine the Adaptability of the Staircase Phenomenon to the Assay of Cardiac Glycosides*

By ARMAND C. BROUSSEAU,† MYRON R. SEGELMAN,‡ and HOWARD J. JENKINS

Results of the investigation presented herewith point to the possibility of the use of the staircase abolition response in the specialized assay of cardiac glycosides and provide at the same time a method potentially applicable to research directed toward the discovery of more effective cardiotonic substances.

ALTHOUGH MANY INVESTIGATORS since the discovery of the staircase phenomenon by Bowditch (1) have attempted to determine the underlying principle involved in this response (2–9), few have been concerned with its practical application. There is the possibility, advanced in this study, that the response may serve not only to quantitate cardiotonic potency but also, in so doing, to provide information which may be used in conjunction with the earlier work to establish the mechanism of the action of the digitalis group of drugs.

The immediate aim of the study reported in this paper is to determine the adaptability of the staircase phenomenon to the assay of the cardiac glycosides on the basis of both reproducibility and specificity. The procedure developed for this determination constitutes, in the opinion of the authors, a step toward the ultimate goal of the study, *viz.*, the production of a device for accurately measuring the potency of small quantities of highly active cardiac glycosides. Such a device, it is felt, could be employed to direct purification programs designed to provide a better "digitalis" and to locate such material if it exists.

EXPERIMENTAL

Preliminary work indicated that the pattern of Dale (7) was the most satisfactory for this work. This pattern presents the staircase as a series of large, descending steps, each formed by the equilibrium amplitude of a particular frequency in the descending stimulation schedule which begins at one stimulus per two seconds and continues as far down as one stimulus per fifteen or twenty seconds.

"Response" was designated by the interval between inciting impulses. A response resulting from a frequency of one stimulus per two seconds was thus considered a two-second response, that from a frequency of one stimulus per four seconds a four-second response. When the four-second response was less than the two-second, it was termed more specifically four-second staircase.

Following the administration of the preparation under study, abolition of staircase was claimed at that response to the lowest frequency which equals or nearly equals in amplitude the response to the highest stimulation frequency in the schedule. For example, that response to the lowest frequency which equals the response to the highest frequency (Fig. 1) is the response at the stimulation frequency of one impulse per ten seconds (ten-second response). This is designated "ten-second staircase abolition," and since a total dose of 2 mcg. of digitoxin accomplished this abolition, we credit this dose of the drug with ten-second staircase abolition. (The abbreviation "std" which appears on the kymograph tracings refers to Reference Standard Digitoxin.)

The experimental portion of the work was concerned as much with the development of a satisfactory procedural scheme as with the determination itself, for without such scheme the question of adaptability itself could not be answered. Accordingly, preliminary investigation involved working out the details of proper dosage of drug, the optimal abolition response, dosage method, abnormal sensitivity of individual hearts, etc. Later efforts were devoted to the refinement of the operational technique. The result represents an attempt not only to determine the magnitude of the response error inherent in the staircase phenomenon (used to quantitate cardiac glycoside potency), but also to set forth a method of operation wherein the response error is kept minimal.

To determine variation in the abolition response to constant doses of the same standard preparation, various quantities of preparation were employed as doses and different abolition responses (i. e., four-second, ten-second and fifteen-second staircases) were used as a basis for comparison.

In the proposed operational scheme, single hearts for each determination are specified because there was evidence of an appreciable variation in sensitivity to digitoxin from one isolated heart to another. Employment of the single excised heart for both reference standard and assay preparation obviates error arising from "animal" variation when the two substances are administered to different test units and provides the advantage of comparison on the same tissue under identical conditions within a comparatively short time-span. There is, however, a limit to the extent of the usefulness of single

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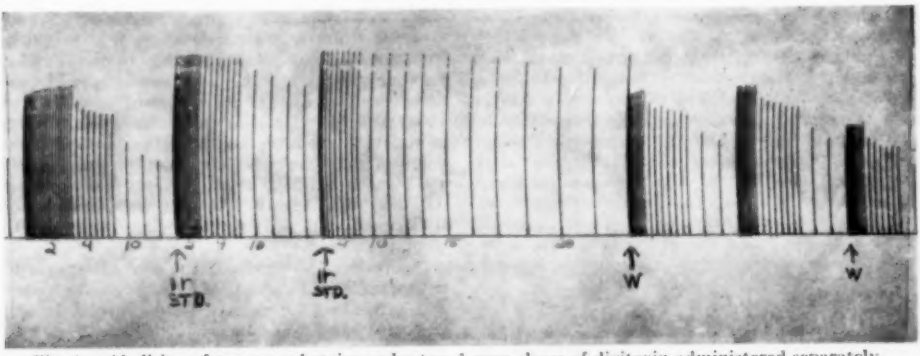

Fig. 1.—Abolition of ten-second staircase by two 1-mcg. doses of digitoxin administered separately.

hearts so employed. It was apparent in this study that the heart never completely recovers from exposure to quantities of digitoxin which are administered over the time period allotted to an individual test. This deleterious effect is noticeable after two, three, or four such doses of digitoxin and two or three intervening washouts. It may be attributable to the digitoxin itself and to the weakening effect upon the myocardium of repeated washing. Whatever its cause, it becomes an increasing source of error as the number of doses of digitoxin and the number of intervening washings are increased.

To decrease response error, both the quantity per dose of drug to which the heart was exposed and the number of washings were decreased. This was accomplished by introducing a low dose of drug in the fixed volume (3 ml.) of the physiologic solution in the cannula chamber after determining the basic staircase pattern. Following a specific exposure period of ten minutes (during which the heart was stimulated every four seconds), the effect of this dose on the pattern was recorded. Then without washing to the reappearance of the basic pattern as previously, a second equivalent dose was added to that already present in the cannula chamber, and a second recording made after an exposure period of five minutes (during which the heart was stimulated once every four seconds). If a third dose was introduced, an exposure period of five minutes (with a four-second stimulation for the period) was again used before recording. In some instances as many as six doses of standard and six doses of drug for assay were administered with only one intervening washing; and the quantity of drug was increased gradually, so that the heart had opportunity to become acclimated to the larger doses.

Two and three series of two and three doses each were employed in the work. The intervals between the doses in each series, including the recording time involved, were practically identical. This obviated any error resulting from variation in time of exposure. The separate doses of digitoxin appear to have an additive rather than a potentiating effect (Figs. 1 and 2) (in Fig. 2, two 1 mcg. doses were administered). Also, the time required for digitoxin to exert its effect is apparently independent of dosage size.

The chief item of equipment was a specialized

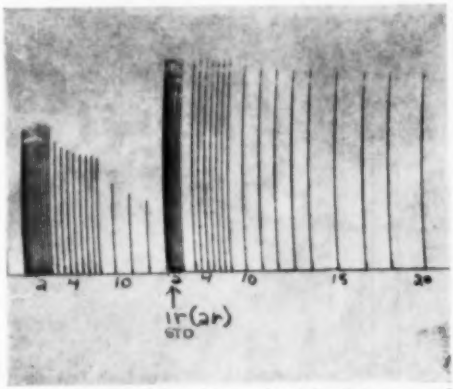

Fig. 2.—Abolition (approximate) of ten-second staircase by two 1-mcg. doses of digitoxin administered together.

cannula designed for the isotonic registration of the heart's contraction, while permitting the contact of administered drug with ventricular tissue through the medium of the physiologic solution contained in the chamber portion (Fig. 3). Through the stainless steel central wire (beaded at either end) of this cannula, the electrical impulse was transmitted to the myocardium. As the latter contracted in response, the central wire, its lower bead resting in the apex of the ventricle, was thrust upwards lifting as it moved the writing lever with which it maintained contact.

The excised heart of the leopard frog, *Rana pipiens*, was immersed in a bath of physiologic solution which was kept at a constant temperature (19°) in a thermos bottle. Lowering the bath temperature tended to abolish the basic staircase pattern. A second electrode was placed in the bath.

Square waves of 5-milliseconds duration were generated by an electronic stimulator at a selected rate. The cannula was held by a burette holder affixed to a rack and pinion. The approximation of cannula wire and writing lever was therefore finely adjustable. A 19-gauge hypodermic needle, attached to water suction, was inserted in the cannula chamber to facilitate washing of the heart.

All clamps were attached to the same supporting

rod, which had a washer and nut at its base. This permitted fine adjustment of the writing point to the drum as a result of the swiveling action achieved.

A modified Boyle-Conway solution (10), the physiologic solution referred to above, was used in both the cannula chamber and the thermos bath. This solution was capable of keeping the isolated heart functional for several days. It had a pH of neutrality. The U. S. P. Reference Standard

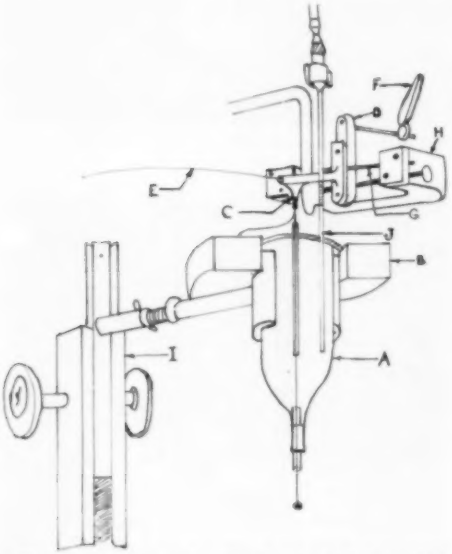

Fig. 3.—A, glass cannula; B, salt water bath (into which connecting wire dips); C, housing for upper bead of central wire; D, aluminum writing arm holder; E, writing arm; F, counterbalance clip; G, watch spring (under tension); H, brass fork; I, rack and pinion; J, 19-gauge needle attached to suction nozzle.

Digitoxin used was kept in a stock solution of propylene glycol. Dilutions reduced it to less than 0.5%, which had no effect on staircase pattern.

The heart's own rhythm was destroyed by cautery of the auricular tissues. This was a very important step in readying the preparation, since the operator must have complete control of the contraction rate at all times.

RESULTS AND DISCUSSION

In the greater portion of the work the error in the reproducibility of the abolition response, at the level of four-second staircase when only the first and second series were involved, was 5% or less (Table I). The highest percentage error at this point was 9.5%; and it plus three other errors over 5% were largely ascribable to rather wide variations in the percentage difference between the two-second and four-second responses prior to the administration of the doses in each of the two series concerned. In other words, there was considerable difference in the pre-dose staircase patterns before each dose series of a single determination (heart washed between series), so that it was difficult here to make a valid comparison of the effectiveness of identical doses of drug in different series with respect to staircase abolition. Whenever the staircase pattern resulting from a switch from one stimulus per two seconds to one stimulus per four seconds was the same, or very nearly the same, prior to each of two series of doses (in the absence of affecting drug in the cannula), the responses obtained on the addition of equivalent doses of digitoxin were the same or very nearly so (Figs. 4 and 5). If the previous staircase patterns were appreciably different, the responses to the addition of equivalent doses of digitoxin tended to be quite different.

Figures 4 and 5 pertain to the error in response to unvarying initial doses of digitoxin in each series. The percentage errors related to constant second and third doses were somewhat higher.

On the basis of the findings of these studies, it would seem advisable to utilize the four-second abolition response as the end point for the comparison of equivalent doses of glycoside preparations and to employ only two series of two or three doses each in the same heart.

Both hypo- and hypersensitivity of hearts to digitoxin were encountered. Since a comparison standard is run with each sample, this would be of little consequence were it not for the fact that the percentage reproducibility error is increased by abnormal sensitivity.

To combat hypersensitivity, in which no staircase pattern results from a switch from one stimulus per two seconds to one stimulus per four seconds, very minute quantities of acetylcholine (hundredths of a mcg.) were used successfully, though no specific quantity seemed suitable for all hearts. Larger quantities of atropine were necessary to abolish hyposensitivity to digitoxin, one drop of a 1% solution achieving this end.

In those instances in which a dose of drug in a series abolished a particular staircase, the ability of the next equivalent dose (additive effect) to abolish

TABLE I.—ERROR INVOLVING THE STAIRCASE ABOLITION RESPONSES TO THE SAME DOSE OF THE FIRST AND SECOND SERIES OF DOSES

Test	Experimental Procedure	% Error at 4 sec. staircase	% Error at 10 sec. staircase
1	Two dose–two series	4.0	0.8
2	"	0.6	12.6
3	"	6.0	2.0
4	"	2.2	4.6
5	"	1.2	1.0
6	"	0.0	2.7
7	"	8.5	16.0
1	Three dose–two series	2.8	6.0
2	"	3.0	2.5
3	"	5.4	6.0
4	"	0.0	18.0
1	Two dose–three series	2.5	8.8
		5.7[a]	8.0[a]
2	"	1.0	0.1
		3.4[a]	6.8[a]
3	"	3.0	17.5
		17.6[a]	25.2[a]
4	"	9.5	—
		6.0[a]	—
5	"	—	3.0
		2.2[a]	11.0[a]
1	Three dose–three series	3.6	8.0
		1.6[a]	16.0[a]

[a] Third series of doses.

the same staircase was assumed, and the stimulation schedule was begun at the frequency whose decrease in amplitude had been abolished by the preceding dose. For example, if four-second staircase was abolished by 1 mcg. of a drug, the effect of the second 1-mcg. dose (in the series) on succeeding staircase responses was determined, beginning the stimulation schedule for the second dose at one stimulus per four seconds (Figs. 4 and 5). This procedure tended to spare the heart.

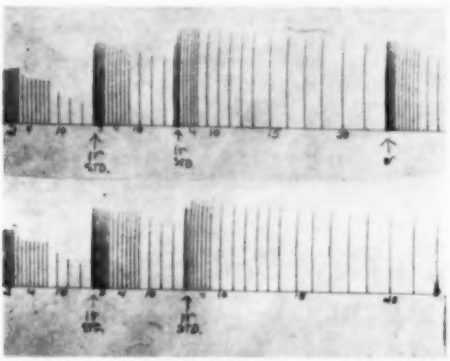

Fig. 4 (above) and Fig. 5 (below).—Identical abolition responses from same dose series when predose staircase pattern is same (consecutive series in same heart).

With respect to specificity, these studies endeavored to provide information as to whether other substances than cardiac glycosides are capable of eliciting the staircase-abolition response, in order to determine the applicability of a method incorporating it. The information obtained appears in Table II.

The testing of synthetic modifications of natural products was resorted to in an effort to get information which might be pertinent to an understanding of the possible activity of metabolic breakdown products of the natural materials.

Those doses appearing in Table II, which were administered to the same heart, represent the totaled values in all but the first listing.

That this method represents a rational approach to the bio-assay of the cardiac glycosides is supported by the following considerations:

Not every agent which increases the amplitude of contraction of the isolated frog heart has the ability to abolish staircase. For example, adrenaline and ATP are capable of amplitude increase but not of staircase abolition. Likewise not every agent which increases the force of the cardiac contraction is capable of restoring compensation in the decompensated heart.

In the isolated frog heart, cardiac glycosides exhibit the dual action of increasing the amplitude of the cardiac contraction while abolishing or tending to abolish staircase. The same dual activity is exhibited in the isolated heart by frog serum, some substance of which apparently prevents the appearance of the staircase phenomenon in the normal heart of the intact frog (the phenomenon is not seen in the normal organ of the intact animal).

Although the staircase-abolition response has been shown in this work to be nonspecific for cardiac glycosides, the method incorporating it, which is advanced in this work, may still be used to seek out a physiologic material in serum with possible digitalis-like activity.

The presence of the staircase-abolishing agents employed in this study, some of which have been shown in other testing to be cardiotonic (11), does not preclude the use of the method herewith presented to search out in serum a possible highly active cadiotonic entity. If these agents are not present in concentrations deleterious to the excised frog heart, it may be assumed that they would exert a synergistic effect with the highly active constituent. Then, as refinement was directed by assay results, the constituent, if present, would gradually come into focus.

The same result would be expected in the use of the method in a search for a better product from crude cardiac glycoside preparations. There may be present in such preparations substances which exert the same qualitative effect as the more highly purified material sought; but so long as they are not antagonistic in any way to the heart, they would not

TABLE II.—EFFECTS OF VARIOUS DOSES OF AGENTS TESTED

Agent	Dose	Four-Second Staircase	Ten-Second Staircase
Estradiol[a]	60.0 mcg.	d	—
	120.0 mcg.	+	—
	180.0 mcg.	+	—
	240.0 mcg.	+	—
	300.0 mcg.	+	—
Ethinyl estradiol	2.5 mcg.	+	+
	5.0 mcg.	r	r
	15.0 mcg.	r	r
	30.0 mcg.	r	r
	60.0 mcg.	r	r
Progesterone[a]	30.0 mcg.	d	—
	60.0 mcg.	+	—
Anhydrohydroxy-progesterone[a]	30.0 mcg.	d	—
	60.0 mcg.	+	—
Testosterone	15.0 mcg.	—	—
	30.0 mcg.	—	—
	60.0 mcg.	d	d
	120.0 mcg.	+	+
Methyltestosterone	10.0 mcg.	—	—
	15.0 mcg.	—	—
	30.0 mcg.	+	d
	60.0 mcg.	r	r
	90.0 mcg.	r	r
Prednisone[a]	30.0 mcg.	—	—
	60.0 mcg.	—	—
	90.0 mcg.	—	—
	120.0 mcg.	—	—
	150.0 mcg.	—	—
Corticosterone[a]	15.0 mcg.	—	—
	30.0 mcg.	—	—
Hydrocortisone[a]	30.0 mcg.	—	—
	60.0 mcg.	—	—
Cholesterol (in acetone)	10.0 mcg.	—	—
	100.0 mcg.	—	—

[a] All doses administered to the same heart. +, Abolition of staircase; d, staircase less pronounced; r, staircase reversed; —, no effect on staircase.

act as contaminants and would not give false values.

The method is not specific for the cardiac glycosides but for the assessment of cardiotonic activity.

SUMMARY

1. The results of studies pertaining to the adaptability of the staircase phenomenon to the assay of cardiac glycosides on the basis of reproducibility and specificity of response are presented.

2. These results indicate that the staircase phenomenon is capable of a high degree of accuracy in potency evaluation when a single excised frog heart is employed for the comparison of reference standard and unknown strengths using no more than two series of two doses each, with the response for comparison set at four-second staircase abolition.

3. The results further indicate that the staircase-abolition response is nonspecific for the cardiac glycosides, but specific for cardiotonic activity.

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A Pharmacological Study of a New Orally Effective Adrenergic Blocking Agent (A–2077)*

By G. VICTOR ROSSI and THOMAS H. F. SMITH

Preliminary pharmacologic evaluation of a new series of benzodioxane derivatives indicated that 2-(N,N-diethyl-carbamylmethyl)-aminomethyl-1,4-benzodioxane (A-2077) possesses low acute toxicity, provides protection against the lethal effects of epinephrine in mice, produces moderate and fairly sustained reduction of blood pressure in anesthetized normotensive cats and dogs following oral and intravenous doses which also inhibit or reverse the pressor response to sympathomimetic amines. This compound appears to be an adrenergic blocking agent with considerable oral effectiveness.

AMONG THE FIRST synthetic substances exhibiting adrenergic blocking activity were the benzodioxane conpounds developed by Fourneau and his collaborators (1). In the large series of compounds which were examined, all degrees of potency and specificity were found to exist. Certain of these derivatives, exemplified by 2-(1-piperidyl methyl)-1,4-benzodioxane (Piperoxan®, 933 F), selectively inhibit the effector cell response to circulating adrenergic mediators, whereas others, such as 2-(N,N-diethyl)-aminomethyl-1,4-benzodioxane (Prosympal®, 883 F), also effectively block the responses to sympathetic nerve stimulation. Although most adrenergic blocking agents appear to be considerably more effective against responses to circulating sympathomimetic agents than against responses to sympathetic nerve stimulation, all degrees of effectiveness against these two stimuli are generally present within each series (2). The usefulness of adrenergic blocking agents of the benzodioxane series in the treatment of hypertension has been severely limited by their lack of potency, specificity, their unpredictable hypotensive effects when administered orally, and numerous nonadrenergic side effects.

The specific purpose of this investigation was to study the adrenergic blocking effectiveness of a new series of compounds of the benzodioxane class when administered orally. The adrenergic blocking ability of this type of compound appears to reside in the benzodioxane nucleus, and while side chain substitutions tend to increase the effectiveness, they also increase the toxicity as they increase in complexity. On the basis of this supposition, a series of 2-N-mono- and di- substituted, 2-methyl-carbamyl-aminomethyl-1,4-benzodioxanes were synthesized (Table 1).

These compounds were subjected to preliminary pharmacological evaluation by an estimation of the intraperitoneal acute toxicity in

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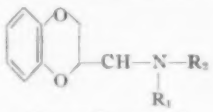
TABLE I.—A SERIES OF NEW COMPOUNDS TESTED FOR ADRENERGIC BLOCKING ACTIVITY



Parent Compound

2-(R,R-Carbamylmethyl)-aminomethyl-1,4-benzodioxane

	Substituted Groups		
	R_1	R_2	Name
1	H	$-CH_2-CO-NH-CH_3$	2-(N-Methyl-carbamyl-methyl)-amino-methyl-1,4-benzodioxane
2	H	$-CH_2-CO-NH-C_2H_5$	2-(N-Ethyl-carbamyl-methyl)-amino-methyl-1,4-benzodioxane
3	H	$-CH_2-CO-NH-CH(CH_3)_2$	2-(N-Isopropyl-carbamyl-methyl)-amino-methyl-1,4-benzodioxane
4	H	$-CH_2-CO-NH-CH_2-CH=CH_2$	2-(N-Allyl-methyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
5	H	$-CH_2-CO-N(C_4H_9)_2$	2-(N,N-Dibutyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
6	H	$-CH_2-CO-N(C_4H_9)(C_2H_5)$	2-(N-Butyl,N-ethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
[a] 7	H	$-CH_2-CO-N(C_2H_5)_2$	2-(N,N-Diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
8	C_2H_5	$-CH_2-CO-NH_2$	2-(N-Ethyl-N-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
9	C_2H_5	$-CH_2-CO-N(CH_3)_2$	2-(N-Ethyl-N,N-dimethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
10	C_2H_5	$-CH_2-CO-N(C_2H_5)_2$	2-(N-Ethyl-N,N-diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
11	C_2H_5	$-CH_2-CO-N(CH_2-CH=CH_2)_2$	2-(N-Ethyl-N,N-diallyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
12	C_2H_5	$-CH_2-CO-NH-C_2H_5$	2-(N-Ethyl-N-ethylcarbamyl-methyl)-aminomethyl-1,4-benzodioxane
13	$CH_3-CH-CH_3$	$-CH_2-CO-N(C_2H_5)_2$	2-(N-Isopropyl-N,N-diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
14	$CH_2-CH=CH_2$	$-CH_2-CO-N(C_2H_5)_2$	2-(N-Allyl-N,N-diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
15	$CH_2-CH=CH_2$	$-CH_2-CO-N(CH_2-CH=CH_2)_2$	2-(N-Allyl-N,N-diallyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane
16	C_2H_5	$-CH-CH-CO-N(C_2H_5)_2$	2-(N-Ethyl-N,N-diethyl-carbamyl-ethyl)-carbamyl-methyl)-aminomethyl-1,4-benzodioxane

[a] Compound A-2077.

TABLE II.—COMPARISON OF THE DEPRESSOR EFFECTS AND ADRENERGIC BLOCKING ACTION OF A-2077 IN DOGS

Dose of A-2077 mg./Kg. and Route of Administration	Number of Animals	Effect on Blood Pressure Mean % Decrease	Mean Duration of Depressor Effect in Minutes	Effect on Pressor Response to Epinephrine —Time in Minutes after A-2077— 30	60	120	180	240
10 *per os*	6	<1	...	I	I	R	R	I
20 *per os*	9	24	150	I	I	R	R	I
50 *per os*	2	28	150	I	R	R	R	I
100 *per os*	5	40	180	R	R	R	R	R
0.5 i.v.	3	<1	...	...				
1 i.v.	3	30	20	I				
5 i.v.	3	70	25	I				
10 i.v.	2	Fatal	...					
50 s.c.	3	50	90	I				

I = 50% Inhibition; R = 50% Reversal.

mice, the depressor effect following oral administration in anesthetized normotensive dogs, and their effect in reducing the pressor response to intravenously injected epinephrine. Based on the results of these preliminary experiments, the compound 2-(N,N-diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane (A-2077)[1] (No. 7 in Table I), showed the most promise and was selected for further study. It is interesting to note that this compound differs from 883F (Prosympal) only by the insertion of a carbamyl-methyl group in the middle of the side chain substitution.

EXPERIMENTAL

Acute Toxicity in Mice.—Male albino mice (Taconic Farms) weighing from 20 to 25 Gm. and fasted for twenty-four hours were used for the determination of oral and intraperitoneal LD_{50}. Those animals which succumbed did so within three hours after administration of A-2077. In each case, death was preceded by tremors, clonic and tonic convulsions. LD_{50} values and their limits of error were calculated according to a modification of the method of Epstein and Churchman (3). The oral LD_{50} of A-2077 was found to be 322 mg./Kg. (95% Fiducial limits of 281 and 367 mg./Kg.), and the intraperitoneal LD_{50} was determined to be 167 mg./Kg. (95% Fiducial limits of 148 and 186 mg./Kg.).

Reduction of Epinephrine Toxicity in Mice.—The degree and duration of protection provided by both oral and intraperitoneal administration of A-2077 against the lethal effect of epinephrine in mice was determined according to the method of Loew and Micetich (4) with minor modification. The doses of A-2077 employed were 80.5 and 161 mg./Kg., *per os* (representing $^1/_4$ and $^1/_2$ of the oral LD_{50}, respectively); 41.7 and 83.5 mg./Kg., i.p. ($^1/_4$ and $^1/_2$ of the intraperitoneal LD_{50}, respectively). These doses were given in a constant volume of water (*per os*) or saline (i.p.) to groups of 10 male albino mice (20 to 25 Gm.; fasted twenty-four hours). At varying time intervals after the administration of the adrenergic blocking agent, epinephrine was injected intraperitoneally in a dose of 14.4 mg./Kg., which sufficed to kill 67% of the unprotected (saline injected) control mice. A group of 10 control mice was injected with the standard dose of epinephrine at each time interval and the results of these experiments were pooled to obtain the average per cent mortality as indicated by the dotted line in Fig. 1.

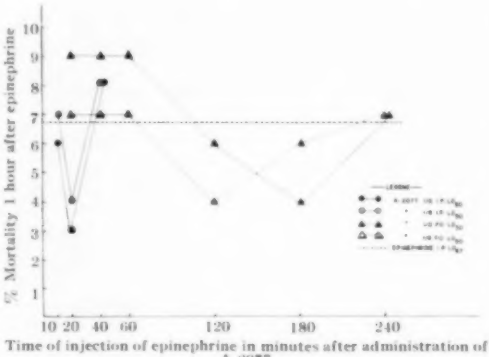


Fig. 1.—Antagonism of epinephrine toxicity in mice by A-2077 2-(N,N-diethyl-carbamyl-methyl)-aminomethyl-1,4-benzodioxane.

This value is in agreement with that obtained by Loew and Micetich (4).

Administration of both $^1/_4$ and $^1/_2$ the oral LD_{50} of A-2077 provided marked protection against the lethal effects of epinephrine at both the 120 and 180-minute test period. Maximal protection was offered at 120 minutes by $^1/_4$ the oral LD_{50} whereas maximal effect with $^1/_2$ the oral LD_{50} was not obtained until the 180-minute test period. At the 240-minute test period, this blocking action was dissipated and the toxicity of the epinephrine returned to the control level.

Following intraperitoneal administration of A-2077 a more rapid onset of action was observed, giving protection at the 20-minute test period, but the duration of the effectiveness was considerably shorter.

Blood Pressure Reducing Effect in Normotensive Animals by Various Routes of Administration.—This investigation was primarily concerned with evaluation of the oral effectiveness of A-2077 although subcutaneous and intravenous routes of administration were also employed. Dogs (6 to 8 Kg., both sexes) were anesthetized with pento-

[1] Supplied by Dr. Gustav J. Martin, Research Division, National Drug Co.

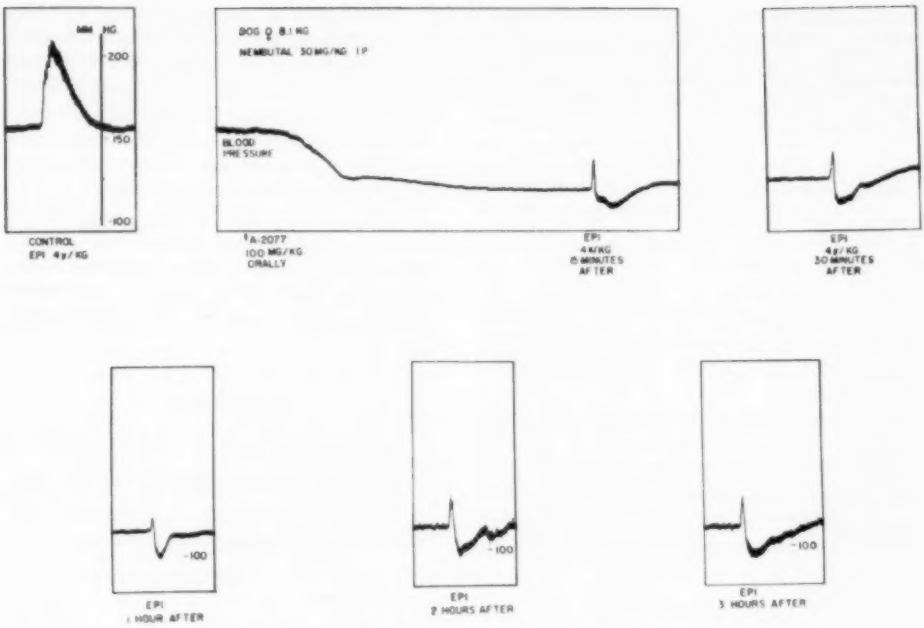


Fig. 2.—The effect of A–2077 on blood pressure and the pressor effect of epinephrine.

barbital sodium (30 mg./Kg., i.p.) and the blood pressure was recorded from the carotid artery. A stomach tube was used for the oral administration of the drug, and the femoral vein was cannulated to permit intravenous injection of other substances. The mean depressor response obtained in dogs for each dose level and the various routes of administration employed are presented in Table II.

Doses of 20, 50 and 100 mg./Kg., *per os*, produced significant reduction of blood pressure, ranging from 11 to 70% of the pretreatment values. Doses of 10 mg./Kg. or less orally produced no appreciable decrease in blood pressure. In the dog, intravenous doses of 1 to 4 mg./Kg. elicited comparable depressor effects; the response to 5 mg./Kg. was somewhat more pronounced and 10 mg./Kg. proved fatal. The duration of the depressor response following oral administration was found to be similar for all doses of A–2077 producing this effect (150 to 180 minutes). By the intravenous route the depressor effect was transient. A dose of 50 mg./Kg. of A–2077 given subcutaneously produced a 50% depression in blood pressure for an average of ninety minutes.

In cats, anesthetized with pentobarbital sodium (25 mg./Kg., i.p.), A–2077 was administered only by the intravenous route in doses ranging from 1 to 8 mg./Kg. These amounts produced no significant depressor effect, with the exception of one animal. As in the dog, the 10 mg./Kg. dose intravenously proved fatal.

Alteration of the Response to Intravenously Injected Sympathomimetic Amines and Other Sympatho-Adrenal Stimuli.—This study was performed with the same animals employed in the investigation of the blood pressure reducing effect of A–2077. Control doses of *l*-epinephrine and *l*-norepinephrine were injected intravenously prior to the administration of the adrenergic blocking agent and the effect noted when the same doses were administered at various time intervals after A–2077 had been given.

Both oral and intravenous doses of A–2077 produced an initial inhibition of the pressor response to epinephrine, and later further modified this response by causing a 50% reversal, i.e. the pressor effect preceding and equal to the depressor effect. Table II lists the average period of time during which A–2077 produced this effect for each of the various dose levels employed. A typical pattern of the effect of A–2077 on blood pressure and the pressor response to epinephrine is shown in Fig. 2. Similar results were obtained with *l*-norepinephrine. The blockade produced by this compound appears to be of the type described by Nickerson (5) as an "equilibrium blockade," in that it can be abolished by large doses of epinephrine. It was further observed that the depressor response following the injection of isopropylarterenol was not significantly altered by A–2077.

Other responses to sympatho-adrenal stimulation such as the nicotinic pressor action of a large dose of acetylcholine in the atropinized animal and the reflex pressor responses to carotid pressoreceptor stimulation by bilateral occlusion for one minute were also examined. It was found that 20 mg./Kg. of A–2077, *per os*, was capable of inhibiting in some cases, and reversing in others, the nicotinic pressor action of acetylcholine. The carotid pressor reflex was effectively blocked by doses as low as 10 mg./Kg. orally.

In cats, intravenous injection of various doses of

A-2077 had no effect on the stimulation of the nictitating membrane by epinephrine or *l*-norepinephrine. A dose of 5 mg./Kg. of A-2077, i.v., effectively blocked the pressor response to these sympathomimetic amines on blood pressure, but showed no indication of a reversal effect.

SUMMARY

1. A new series of 2-N-mono and di-substituted 2-methyl carbamyl-aminomethyl-1,4-benzodioxanes were subjected to preliminary pharmacologic evaluation. On the basis of low acute toxicity, effect on blood pressure and especially oral effectiveness, the compound 2-(N,N-diethyl carbamyl-methyl)-aminomethyl-1,4-benzodioxane (A-2077) was selected for further study.

2. The oral and intraperitoneal LD_{50} values for A-2077 were determined in mice.

3. Oral and intraperitoneal doses of A-2077 were found to offer significant protection against the lethal effect of epinephrine in mice. The times of peak activity were estimated.

4. In a range of oral and intravenous doses, A-2077 produced a moderate reduction of blood pressure in anesthetized normotensive animals (dogs, *per os* and i.v.; cats, i.v.). Both oral and intravenous administration effectively inhibited or reversed the pressor response to intravenously injected sympathomimetic amines and other forms of sympatho-adrenal stimulation.

5. The effect of the carbamyl group in enhancing the oral activity of this series of benzodioxane adrenergic blocking agents indicates the potential value of this group in the further development of orally effective therapeutic agents.

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Studies on the High Voltage Electrophoresis of Strychnos Alkaloids*

By WILLIAM G. GORMAN† and RUPERT SALISBURY‡

The pH of the initial solution of brucine and strychnine affected the electrophoretic migration, with maximum migration occurring near a pH of 6.0 for strychnine and between 5.0 and 6.0 for brucine. The pH of the solvent used to fill the cell also affected the migration of strychnine. The optimal pH for this solvent was near 6.0. Increasing the voltage increased the rate of migration of both alkaloids. Strychnine migrated more readily when introduced nearer to the cathode. The concentration of the initial solution affected the migration of strychnine and brucine. Migration occurred best when high concentrations were introduced, tapering off slightly until at low concentrations the migrations were greatly reduced. The migration of strychnine was affected by the salt anion present, which occurred in the following order: sulfate ≥ hydrochloride > phosphate > lactate.

LITTLE INFORMATION is available on the high voltage electrophoresis of the medium molecular weight compounds which are most frequently encountered in drug and medicinal compounds. Since high voltage electrophoresis appears to possess great possibilities in the separation and isolation of such compounds (1), it would seem important to investigate the fundamental factors involved. It was the object, therefore, to determine the effect of such factors as: pH, voltage, concentration of the solute, site of introduction, salt anion, and time of the electrophoretic migration of strychnine and brucine in the hope that the information gained in this study may be useful in predicting the effects that these variables have on other medicinal products. High voltages were used with nonreversible electrodes to establish a pH gradient and to provide for rapid migration since, as reported by Abramson, Moyer, and Gorin (2), the velocity of a charged particle is directly proportional to the applied voltage.

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Abstracted from a thesis presented to the Graduate School, Ohio State University, in partial fulfillment of the requirements for the degree of Master of Science.
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‡ Assistant Professor of Pharmacy, Ohio State University, College of Pharmacy, Columbus.

EXPERIMENTAL

Assay of Strychnine and Brucine.—Pure anhydrous strychnine, melting point 285°–290°, and

anhydrous brucine, melting point 178°, were used throughout this investigation. A spectrophotometric assay was used for the quantitative determination of both alkaloids. Strychnine was found to exhibit maximum absorption at a wavelength of 254 millimicrons, while brucine exhibits maximum absorption at 264 millimicrons. These values compare favorably with those reported by Bhattacharya and Ganguly (3), Biggs (4), and El Ridi and Khalifa (5). Concentration curves were made that followed Beer's law. Other aspects of the assay were carried out using standard procedures.

General Methods.—The cell used throughout this investigation was that devised by Salisbury and Guth (1). It consists of a series of five V-tubes or segments numbered I–V from anode to cathode. The power supply unit was a Signal Corps Field Rectifier RA-38, capable of supplying up to 15,000 volts d. c.

Migration studies were carried out by filling the cell with distilled water and then introducing an aqueous solution of the alkaloid into the cell through a length of fine vinyl-plastic tubing attached to a hypodermic syringe. A high voltage potential was then applied for a controlled period of time. Temperature and amperage readings were taken periodically. When the current was turned off, the cell segments were drained and collected separately. These fractions were tested for pH and then assayed for their alkaloid content. The bottom of the initial segment was collected separately since any alkaloid that was in the neck above the stopcock was not in the electrical field and would not migrate. The amount of alkaloid found present in the segments other than the initial segment was considered to be the amount that had migrated from the initial segment. The total amount of alkaloid introduced minus the amount in the neck above the stopcock in the initial segment was considered as the amount of alkaloid introduced into the electrical field. Dividing this value into the amount of alkaloid that had migrated from the initial segment, we obtain the per cent migration.

TABLE I.—pH OF EACH SEGMENT WHEN pH OF ORIGINAL SOLUTION IS VARIED

	Average pH Values for Each Segment				
Strychnine Introduced at pH	Segment I	Segment II	Segment III	Segment IV	Segment V
1.0	4.6	4.2	1.8	5.8	8.2
2.0	4.8	4.7	2.3	5.7	8.2
3.0	4.8	4.8	3.1	6.3	8.6
4.0	4.6	4.6	4.1	8.4	9.1
5.0	4.7	4.8	4.8	8.4	9.0
6.0	4.9	5.4	5.8	8.6	9.2
6.5	4.5	4.7	5.6	9.0	9.3
Brucine Introduced at pH					
1.0	3.9	3.2	1.8	6.1	8.1
2.0	4.3	3.5	2.3	5.9	8.1
3.0	4.7	4.5	3.2	5.8	8.7
4.0	4.6	4.6	4.0	8.2	9.0
5.0	5.0	4.9	5.1	8.5	9.2
6.0	4.6	4.6	5.8	8.6	9.2
7.0	4.4	4.6	6.7	8.9	9.4

Effect of pH.—Strychnine lactate solutions (0.0215 *N*) were prepared with pH values of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 6.5, using lactic acid or ammonium hydroxide in the smallest possible amounts. The cell was filled with distilled water (pH 6.8) and 5 ml. of each solution were introduced separately into segment III and a 5,000-volt potential applied for one hour at which time the segments were drained and assayed. The same procedure was repeated for brucine solutions (0.0215 *N*) at pH values of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0. The pH of the initial solution appears to have a profound effect on the resultant migration for both alkaloids as shown by the graph on Fig. 1.

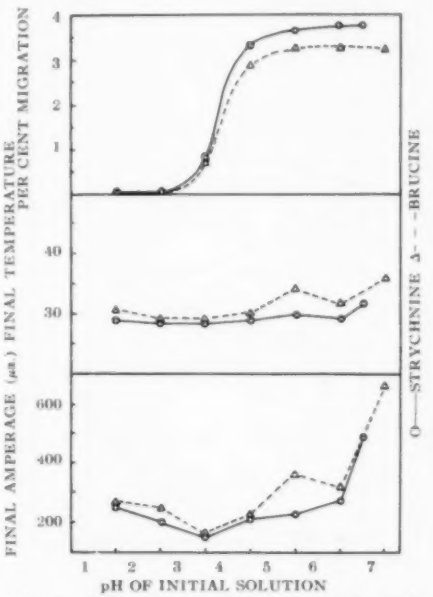

Fig. 1.—Effect of the pH of the initial solution on the electrophoretic migration of strychnine and brucine.

Temperature changes remained rather constant throughout and appear to have little effect in this study.

Since maximum migration appears to occur when the pH of an initial solution of strychnine is about 6.0, 5 ml. of a 0.0215 *N* strychnine lactate solution (pH 6.0) were introduced into the cell filled with water at pH values of 4.0, 5.0, 6.0, 6.8, and 7.0. A 5,000-volt potential was applied for one hour in each case and the segments drained and assayed.

Crystals formed on the walls of Segment III when solvents at pH 4.0 and 7.0 were used. The crystals were collected and found to have a melting point characteristic of strychnine and to form "envelope" micro-crystals with platinic chloride confirming their identity as strychnine. As shown by Table II, the crystals formed between an acid and an alkaline segment. Since strychnine is only slightly soluble in a neutral or alkaline medium, it is logical that crystals should form as the concentration increases in this region. Since the pH gradient is similar in the case of all of the solvents used, the optimum pH was chosen as the pH giving maximum migration with

TABLE II.—pH VALUES FOR EACH SEGMENT WHEN pH OF ORIGINAL SOLVENT IS VARIED

pH of the Solvent (Strychnine)	Segment I	Segment II	Segment III	Segment IV	Segment V
4.0	3.7	3.3	4.0[a]	9.4	9.9
5.0	3.7	4.1	4.6	9.2	9.6
6.0	3.9	4.1	4.8	8.9	9.6
6.8	4.9	5.4	5.8	8.6	9.2
7.0	3.9	4.2	4.9[a]	9.2	9.8

[a] Crystals formed on wall.

TABLE III.—pH OF EACH SEGMENT WHEN VOLTAGE IS VARIED

Voltage (Strychnine)	Average pH Values for Each Segment: Segment I	Segment II	Segment III	Segment IV	Segment V
1000	5.7	6.3	6.0	6.7	7.1
2500	4.9	5.2	6.0	8.4	9.0
5000	4.9	5.4	5.8	8.6	9.2
7500	4.4	4.7	5.3	9.0	9.4
10000	4.2	4.5	4.6	9.3	9.5
12500	3.8	4.4	4.2	9.1	9.5
(Brucine)					
2500	4.8	4.8	6.0	7.2	9.0
5000	4.6	4.6	5.8	8.6	9.2
7500	4.4	4.5	5.3	8.8	9.3
10000	4.1	4.3	5.0	9.2	9.4
12500	4.0	4.1	4.8	9.2	9.5

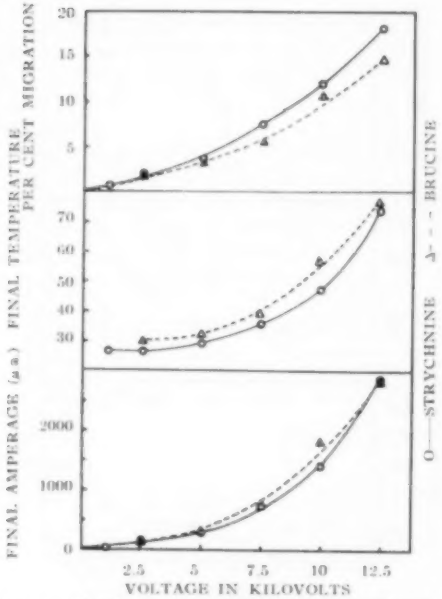

Fig. 2—Effect of voltage on the electrophoretic migration of strychnine and brucine.

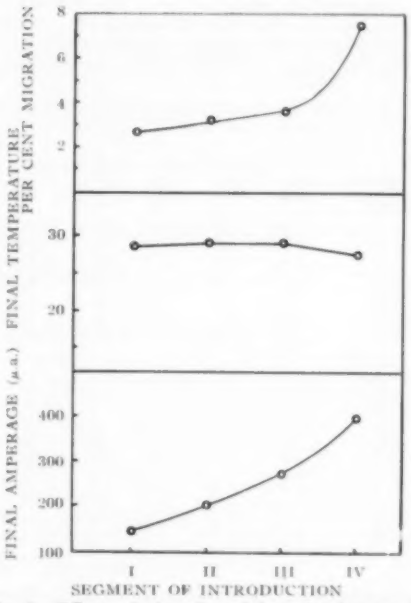

Fig. 3—Effect of the site of introduction on the electrophoretic migration of strychnine.

minimum precipitation, which was about 6.0 for strychnine.

Effect of Voltage.—Five ml. of a 0.0215 *N* strychnine lactate solution (pH 6.0) were introduced into segment III of the cell using distilled water as the solvent within the cell. A potential of 1,000 volts was applied for one hour and the segments then drained and assayed. This procedure was repeated with potentials of 2,500; 5,000; 7,500; 10,000; and 12,500 volts. This procedure was again repeated using 0.0215 *N* brucine solutions. The % migration increased beyond a linear relationship as shown by the graph on Fig. 2. The temperature and amperage both follow similar increases and it is possible that they affect the rate of migration.

Effect of the Site of Introduction.—Five ml. of a 0.0215 *N* strychnine lactate solution (pH 6.0) were introduced into segment I. A potential of 5,000 volts was applied for one hour and the segments were then drained and assayed. This procedure was repeated introducing the solution into segments II, III, and IV. There appears to be a considerable increase in the % migration as the solution is introduced nearer to the cathode. The temperature remained rather constant, but the amperage followed a somewhat similar pattern as the % migration as shown by the graph on Fig. 3. This seems to indicate that the amperage may have some role in affecting the rate of migration, at least in regard to some compounds in this molecular weight range.

Effect of Solute Concentration.—Strychnine solutions of various concentrations, but all at the same pH (6.0), were introduced into segment III in 5-ml. volumes. A potential of 5,000 volts was then applied for one hour after which the segments were drained and assayed. Concentrations of 28.8, 144, 288, 432, 576, and 2,000 milligrams % were used. The same procedure was repeated with brucine using equivalent normal concentrations. As shown by

TABLE IV.—pH OF EACH SEGMENT WHEN SITE OF INTRODUCTION IS VARIED

Strychnine Site of Introduction					
Segment I	5.4	6.8	7.3	8.4	9.2
Segment II	4.6	5.5	7.9	8.6	9.3
Segment III	4.9	5.4	5.8	8.6	9.2
Segment IV	4.7	4.9	5.5	5.5	9.0

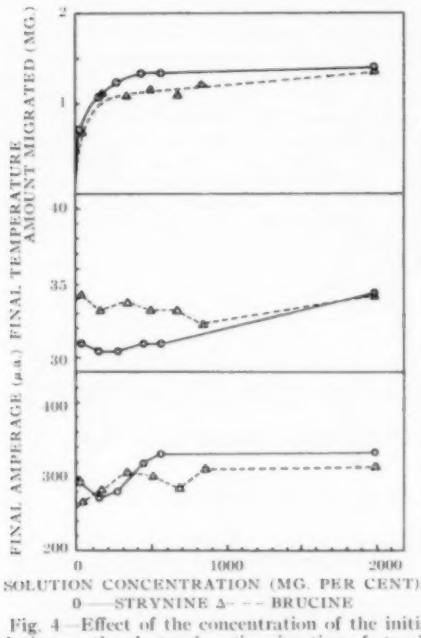

Fig. 4—Effect of the concentration of the initial solution on the electrophoretic migration of strychnine and brucine.

the graph on Fig. 4, the concentration of the initial solution has only a slight effect on the amount of migration at the higher concentrations, while this effect is quite pronounced at the lower concentrations.

Effect of the Salt Anion.—Strychnine alkaloid was added to distilled water containing enough lactic acid to react with the base. The resultant solution was adjusted to pH 6.0 with dilute ammonium hydroxide. This solution was then diluted to a 0.0215 *N* concentration using distilled water adjusted to pH 6.0 with lactic acid. Five ml. of this solution were introduced into segment III and a potential of 5,000 volts was applied for one hour. The segments were then drained and assayed. This procedure was repeated using sulfuric, hydrochloric, and phosphoric acids in place of the lactic acid. The graph on Fig. 5 shows the % migration, final amperage, and final temperature readings in each case. The salt anions appear to affect the migration in the following order: sulfate ≥ hydrochloride > phosphate > lactate.

Effect of Time.—Ten ml. of a 2% solution of strychnine lactate (pH 6.0) were introduced into

TABLE V.—pH OF EACH SEGMENT WHEN CONCENTRATION IS VARIED

Concentration (mg. %) (Strychnine)	Segment I	Segment II	Segment III	Segment IV	Segment V
28.8	4.7	4.7	4.9	8.1	9.4
144	4.7	4.8	5.0	8.6	9.2
288	4.6	4.6	5.3	8.7	9.3
432	4.5	4.5	5.5	8.6	9.3
576	4.5	4.8	5.5	8.0	9.2
2000	4.5	4.7	5.2	8.7	9.1
(Brucine)					
33.9	4.6	4.8	5.3	8.0	9.2
170	4.6	5.1	5.6	8.5	9.1
339	4.6	4.8	5.9	8.8	9.0
509	4.7	4.7	5.9	8.2	9.1
678	4.6	4.8	6.0	8.7	9.2
848	4.6	4.6	5.8	8.6	9.2
2000	4.7	4.8	6.0	8.5	9.1

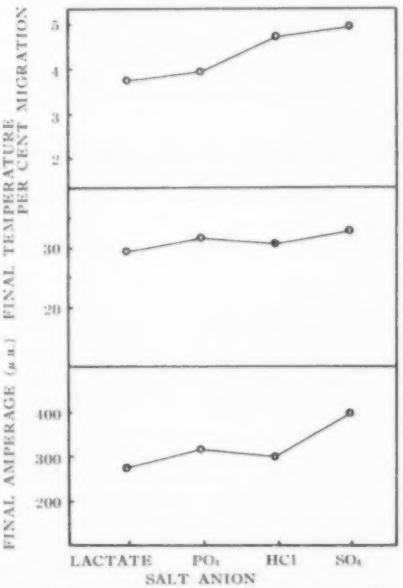

Fig. 5—Effect of the salt anion on the electrophoretic migration of strychnine.

segment III. The bottoms of segments III and IV were filled with mercury to prevent any strychnine from being trapped there. A potential of 5,000 volts was then applied, and segment V was drained periodically and refilled with distilled water. After a total of forty-nine hours of electrophoresis time, the entire cell was drained and the contents assayed. Temperature and amperage changes appear significant, but their erratic patterns make it difficult to interpret their effect. Migration increases to a maximum, levels off, and then becomes rather constant. Any crystals that separated from solution in this study were redissolved in dilute acid

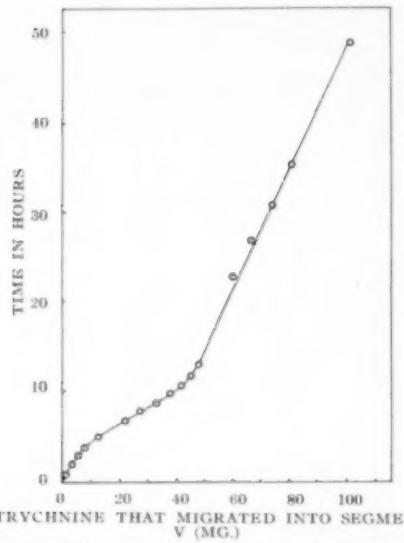

Fig. 6—Effect of time on the electrophoretic migration of strychnine.

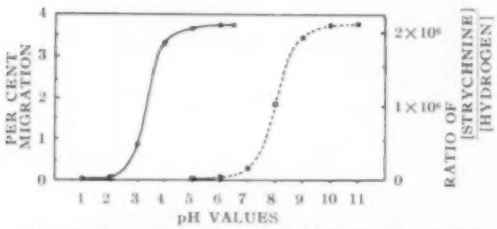

Fig. 7—Comparison of the % migration of strychnine and the ratio of strychnine ions to hydrogen ions at various pH values.

TABLE VI.—EFFECT OF SALT ANION ON THE pH OF EACH SEGMENT AFTER ONE HOUR

Salt anion	Segment I	Segment II	Segment III	Segment IV	Segment V
Strychnine lactate	4.9	5.4	5.8	8.6	9.2
Strychnine phosphate	4.5	5.0	6.0	8.8	9.2
Strychnine hydrochloride	3.9	3.8	4.4	6.9	9.0
Strychnine sulfate	4.2	4.6	4.7	8.8	9.1

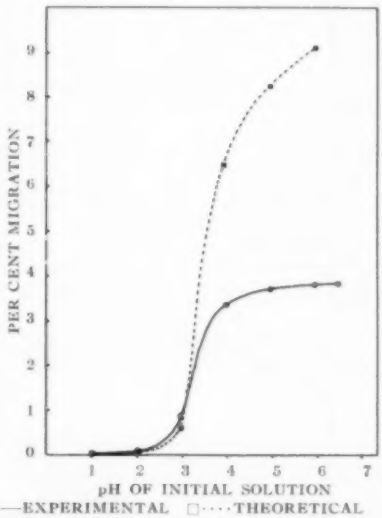

Fig. 8—Experimental values and theoretical estimations of the % migration of strychnine.

and assayed. The amounts of strychnine that migrated into segment V during the various time intervals are shown on the graph,on Fig. 6.

DISCUSSION

The effect produced by pH is quite profound and is believed to influence migration due to its effect on ionization. Using a modified Henderson-Hasselbalch equation

$$pH = pK_a + \log (\text{Base}/\text{Ionized Base})$$

and the pK_b values from Merck Index (6), which are 6.0 and 6.04 for strychnine and brucine respectively, the concentration of ionized alkaloid was calculated for each pH value.

As pointed out by Svensson (7), if large amounts of acids or alkalies collect in the end or neighboring chambers, only a small part of the current is carried by the material in question since the greatest part of the current is carried by the hydrogen and hydroxyl ions and their counter ions. The hydrogen ion concentration was calculated for each pH value and was compared on a molar basis with the concentration of alkaloid ion at the same pH. If the ratio of alkaloid ions to hydrogen ions is plotted against pH a curve is obtained similar to the % migration curve on Fig. 1, but shifted so as to occur at higher pH values. A graph showing this relationship is shown on Fig. 7.

Considering the current available for migration and assuming that all of the hydrogen ions will migrate before the strychnine ions (since the hydrogen ions have a much greater mobility than the strychnine ions) a theoretical approximation of the % migration can be calculated. A comparison of this theoretical value and the experiment % migration is shown on Fig. 8.

As the voltage was increased, the resulting increase in migration above and beyond a linear relationship might be due to temperature or amperage changes. Heating may increase migration by decreasing density of the medium. Convection currents may also aid or abet migration. Attempts were made to use a water bath for cooling, but at these high voltages the current appeared to pass through the cell and be grounded by the bath.

Possibly refrigerated air or a circulating nonconductor would prove adequate in this respect.

Migration occurred at a maximum when the material was introduced nearer to the cathode. Possibly the shorter distance required for the electrical transport offers less resistance to the passage of the current. The increase in amperage in this respect seems to indicate there may be some relationship between the amperage and migration. However, Faraday's law shows no apparent correlation in this respect.

The effect of the concentration of the initial solution on migration might be explained on the basis of competition between alkaloid and hydrogen ions. At high concentrations sufficient alkaloid ions are present for migration to occur at a near maximum rate. Since the hydrogen ion concentration is constant at a given pH, low concentrations of alkaloid decrease the number of alkaloid ions competing with hydrogen ions for the current and the resulting alkaloid migration is decreased

The salt anion present also affected the migration of strychnine. These effects may be due to differences in ionization of the various salts. A study of the transference numbers of these alkaloids would be a great help in understanding the nature and relationship of these effects.

The formation of crystals, as in the study on the effect of time, appears to be due to the pH of the media and the concentration of the solute, since at an alkaline pH solubility of the alkaloids becomes difficult.

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Effect of Flavors upon the Stability of Penicillin in Oral Penicillin-Triple Sulfonamide Powders*

By WALTER F. CHARNICKI and MARY L. KOBER

A number of flavors were screened for their ability to mask the bitterness of an oral powder of potassium penicillin G with triple sulfonamides. The powder, when restored with sufficient water to yield 5 ml. of suspension, contained 100,000 to 250,000 units of penicillin and a mixture of sulfamerazine, sulfadiazine, and sulfamethazine totaling 0.5 Gm. The flavored powders thus selected were subjected to an accelerated penicillin stability test of heating at 100° for four days. Several flavors were selected on the basis of the accelerated test and were used to formulate preparations for long-term stability studies.

MANY ORAL PREPARATIONS of penicillin G with triple sulfonamides are marketed as powders that are reconstituted with water prior to use. The penicillin in this type of a mixture has been found to be very stable (1), and the Food and Drug Administration will grant a thirty-six-month dating to similar marketed products with submission of sufficient evidence of stability (2). In the development of such a dosage form it was discovered that the stability of the penicillin depended greatly upon the flavors used. A survey of the literature showed that flavoring of penicillin G salts has been reported (3), but little could be found concerning the stability of penicillin when combined with various flavors. This paper is the report of such a study in the development of oral preparations of penicillin G with triple sulfonamides.

EXPERIMENTAL

The basic ingredients used in this study consisted of potassium penicillin G, U. S. P., sulfamerazine U. S. P., sulfadiazine U. S. P., sulfamethazine U. S. P., a suspending agent, a surface active agent, saccharin sodium U. S. P., buffer, and sucrose U. S. P. The formula was designed so that each 3.5 Gm. of powder would contain 0.5 Gm. of total sulfonamides and 100,000 units of penicillin. In the tasting and accelerated testing a 10% excess of penicillin was used. In the subsequent work this was raised to a 35% excess. In two combinations the penicillin unitage was raised to 200,000 units plus a 10% excess and 250,000 units plus a 20% excess, and the total quantity of powder reduced to 2.5 Gm., everything else remaining the same. These powders were restored with enough water to yield 5 ml. of suspension having a pH of 6.2–6.5.

The penicillin was assayed by the official plate assay method of the Food and Drug Administration (4), the accuracy of which is considered to be approximately ±10%. Samples were restored with

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the proper amount of distilled water and diluted accordingly.

The flavors employed in these preparations included those official in the U. S. P. XIV and N. F. IX as well as flavor concentrates supplied by the following manufacturers: Fritzsche Brothers, Inc. (Fr); Firmenich, Inc. (Fir); Dodge and Olcott, Inc. (DO); Magnus, Mabee, and Reynard, Inc. (MMR); Roure-Dupont, Inc. (RD); Sharp and Dohme (SD); P. R. Dreyer, Inc. (Dr); and The Dow Chemical Company (Dow). The percentage of flavors used was on a volume to volume basis unless otherwise indicated.

Screening of Flavors for Taste.—The purpose of this screening was to eliminate all flavors for future work which did not aid in masking the bitterness of the penicillin and the sulfonamides. The flavors were incorporated by first restoring the powder and then adding the flavors to the suspension. The amount of flavor to be used in the screening was determined by the authors who tasted suspensions containing different concentrations of each flavor and submitted to a panel that strength of flavored suspension thought to be most effective. Five milliliters of each flavored suspension was tasted by a panel of five fellow research associates. The panel members were asked to indicate whether the flavored preparations were poor, fair, or good. Only those which were considered to be poor were eliminated. In order to avoid the loss of taste sensitivity, only three to five flavored suspensions were given to the panel members at any one time. Sampling was restricted to one morning and one afternoon session. The flavors and the concentrations tried with 100,000 unit, 200,000 unit, and 250,000 unit penicillin-triple sulfonamide suspensions are shown in Tables I and II.

Accelerated Stability Tests of Flavored Preparations.—Penicillin-triple sulfonamide powders containing flavors thought to have any merit by the screening panel in masking the bitterness were subjected to an accelerated stability test. The accelerated test outlined by the Food and Drug Administration consists of heating crystalline penicillin for four days at 100°, followed with assays for penicillin (5). A modification of this test was used in these studies in which the crystalline penicillin was replaced by individually flavored penicillin-sulfonamide powders. At the end of the four days the powder was restored with the proper amount of water and assayed for penicillin activity. The moisture content of the powders used in these studies ranged from 0.07% to 0.44%, which is well below the 1% limit of the Food and Drug Administration (2).

The preparations containing 100,000 units of penicillin per 3.5 Gm. were flavored by dissolving the flavors in 4% (v/w) of acetone and then incorporating the solution into the powder using a mortar and pestle. Exception to this was the imitation maple flavour (Fir), which was added as such, since it was not soluble in either acetone or absolute alcohol.

The use of acetone in the flavoring of the powders appeared to have some detrimental effect upon the suspension qualities of the reconstituted preparations; therefore, the penicillin-triple sulfonamide mixtures containing 200,000 units and 250,000 units of penicillin per 2.5 Gm. were flavored by dissolving the flavors in 5% (v/w) dehydrated alcohol N. F. and mixing with the powder in a mortar and pestle. Exceptions to this were the Fritzbro Arome strawberry imitation (Fr), and the dry flavors vanillin U. S. P., coumarin N. F., Palatone® (Dow), and Cyclotene® (Dow), which were added and mixed by geometric addition.

After the flavors were incorporated, the powders were spread out on paper and air-dried at room

TABLE I.—FLAVORS USED TO MASK TASTE OF 100,000-UNIT PENICILLIN-TRIPLE SULFONAMIDE POWDER

	Flavor	Concentration, %
1.	Clove oil, U. S. P.	0.03
2.	Magna imitation honey flavor,[a] (MMR)	0.40
3.	Imitation cherry Morello flavour, (Fir)	0.002
4.	Imitation heliotrope, (Fir)	0.002
5.	Imitation banana flavour, (Fir)	0.002
6.	Imitation apricot flavour, (Fir)	0.002
7.	Methyl salicylate, U. S. P.	0.04
8.	Pineapple, (Fir)	0.50
9.	Imitation cherry flavor,[a] (MMR)	1.50
10.	Magna imitation pineapple flavor, Pineapco type, (MMR)	1.50
11.	Imitation maple flavour,[a] (Fir)	0.50
12.	Anise oil, U. S. P.	0.02
13.	Imitation spiced peach, (Fir)	0.05
14.	Pineapple, (Fir)	0.015
	Clove oil, U. S. P.	0.005
15.	Pineapple, (Fir)	0.04
	Magna imitation honey flavor, (MMR)	0.40
16.	Clove oil, U. S. P.	0.03
	Magna imitation honey flavor, (MMR)	0.40
17.	Tetrarome lemon, (Fir)	0.50
	Imitation vanilla flavour, (Fir)	0.25
18.	Sassafras oil, U. S. P.	0.03
	Imitation maple flavour, (Fir)	0.02
19.	Fritzbro superarome imitation raspberry, (Fr)	0.05
	Oil sweet orange sesquiterpeneless, (Fr)	0.01
20.	Oil sweet orange sesquiterpeneless,[a] (Fr)	0.005
	Imitation blood orange flavour, (Fir)	0.005
	Essence peach blossom, (RD)	0.005
21.	Imitation maple flavour, (Fir)	0.50
	Imitation walnut green flavour, (Fir)	0.005
22.	Anise oil, U. S. P.	0.005
	Imitation maple flavour, (Fir)	0.50
23.	Fritzbro Superarome imitation raspberry,[a] (Fr)	0.05
	Essence peach blossom, (RD)	0.005
24.	Fritzbro Superarome imitation raspberry, (Fr)	0.05
	Oil sweet orange sesquiterpeneless, (Fr)	0.01
	Imitation abbatiale C flavour, (Fir)	0.02
25.	Imitation maple flavour, (Fir)	0.50
	Clove Oil, U. S. P.	0.03
23.	Root beer, (Fir)	0.02
	Imitation cachou, (Fir)	0.002

[a] Selected by the panel for accelerated penicillin stability tests.

TABLE II.—FLAVORS USED TO MASK TASTE OF 200,000-UNIT AND 250,000-UNIT PENICILLIN-TRIPLE SULFONAMIDE POWDERS

	Flavor	Concentration, % 200,000 Unit	250,000 Unit
1.	Soft drink powder flavor imitation wild strawberry, (Fr)	1.0	.....
2.	Fritzbro Arome strawberry imitation,[a] (Fr)	1.0	.....
3.	Fritzbro Arome sweet cherry imitation, (Fr)	0.7	.....
4.	Fritzbro Arome maple imitation, (Fr)	0.5	.....
5.	Fritzbro Arome raspberry imitation, (Fr)	1.0	.....
6.	Fritzbro Arome peach imitation, (Fr)	0.1	.....
7.	Soft drink powder flavor root beer,[a] (Fr)	0.3	0.3
8.	Astoria flavor No. 25,[a] (DO)	0.1	.....
9.	Imitation coconut flavor, (DO)	0.6	.....
10.	Cosmo N.A. flavor imitation root beer, creamy,[a] (DO)	0.2	.....
11.	Cosmo N. A. flavor imitation pineapple, (DO)	0.1	.....
12.	Dolco flavor imitation strawberry, (DO)	0.2	.....
13.	Arome betel,[a] (Fir)	0.1	0.1
14.	Anise oil,[a] U. S. P.	0.1	0.1
15.	Clove oil,[a] U. S. P.	0.1	0.1
16.	Cinnamon oil, U. S. P.	0.1	.....
17.	Myristica oil, U. S. P.	0.075	.....
18.	Anethole, N. F.	0.1	.....
19.	Safrol, (Dr)	0.1	.....
20.	Orange oil, U. S. P.	0.1	.....
21.	Root beer flavor, (SD)	0.2	.....
22.	Essence Fritzbro lime concentrate,[a] (Fr)	0.05	0.1
23.	Ekomo flavor imitation raspberry,[a] (Fr)	0.1	0.1
24.	Oil marshmallow flavor,[a] (Fr)	0.1	0.2
25.	Fritzbro butterscotch imitation No. 1, (Fr)	0.05	.....
26.	Ekomo flavor imitation maple-walnut, (Fr)	0.025	.....
27.	Essence Fritzbro orange concentrate,[a] (Fr)	0.1	0.1
28.	Ekomo flavor imitation maple, (Fr)	0.05	.....
29.	Magna imitation maple flavor extra,[a] (MMR)	0.05	0.5
30.	Vanillin, U. S. P.	0.6 w/v	.....
	Coumarin, N. F.	0.04 w/v	.....
31.	Palatone, (Dow)	0.02 w/v	.....
	Cyclotene, (Dow)	0.02 w/v	.....
32.	Vanillin,[b] U. S. P.	0.5 w/v	0.5 w/v
	Coumarin, N. F.	0.04 w/v	0.04 w/v
	Palatone, (Dow)	0.02 w/v	0.02 w/v
	Cyclotene, (Dow)	0.02 w/v	0.02 w/v
33.	Oil sweet orange sesquiterpeneless,[a] (Fr)	0.025	0.025
	Imitation blood orange flavour, (Fir)	0.025	0.025
	Essence peach blossom, (RD)	0.025	0.025
34.	Clove oil, U. S. P.	.....	0.05
	Anise oil, U. S. P.	.....	0.1

[a] Selected by the panel for accelerated penicillin stability tests.
[b] Selected by the panel for accelerated penicillin stability tests on 200,000-unit penicillin-triple sulfonamide powder only.

temperature for two to four hours. At the end of this time portions of the powders were placed into suitable containers, heated at 100° for four days, restored with the proper amount of water, and assayed for penicillin content. The results of the accelerated stability tests on 100,000-unit, 200,000-unit, and 250,000-unit flavored penicillin-triple sulfonamide powders are shown in Tables III, IV, and V.

Long-Term Stability Studies.—Upon completion of the accelerated stability studies, long-term stability tests were conducted on the 100,000- and 250,000-unit penicillin combinations.

Those flavored penicillin-triple sulfonamide combinations which appeared most promising from the accelerated stability tests were submitted to a non-technical panel of 15. The panel members indicated those flavors which seemed to give the better tasting preparations. The flavor combination selected by the panel for the 100,000-unit penicillin-triple sulfonamide powder consisted of oil sweet orange sesquiterpeneless (Fr) 0.01%, imitation blood orange flavour (Fir) 0.01%, and essence peach blossom (RD) 0.01%. The two flavors chosen for the 250,000-unit penicillin-triple sulfonamide powder were anise oil U. S. P. 0.1% and clove oil U. S. P. 0.1%. These flavors were placed on long-term stability with the penicillin-triple sulfonamide powders.

Three lots of flavored 100,000-unit penicillin-triple sulfonamide powders were prepared and placed on long-term stability testing. At this time the total concentration of sulfonamides was reduced from 0.5 Gm. to 0.25 Gm. Figure 1 shows the results of penicillin stability with 0.25 Gm. total sulfonamides after twenty-nine and forty-three months' storage at room temperature.

The 250,000-unit penicillin-triple sulfonamide flavored powders, along with an unflavored control, were prepared and placed at room temperature and 37° stability stations. Portions of the powders were restored with water periodically and assayed for penicillin content. The results of penicillin stability following forty months' storage are shown in

TABLE III.—RESULTS OF ACCELERATED PENICILLIN STABILITY TESTS ON 100,000-UNIT[a] PENICILLIN-TRIPLE SULFONAMIDE POWDER

	Flavor	% Conc. of Flavor	Units/3.5 Gm. Powder Initial Assay	% of Theoretical	Heated 4 Days at 100°	% of Theoretical
1.	Unflavored	..	95,000	86.36	88,625	80.57
2.	Oil sweet orange sesquiterpeneless, (Fr)	0.01	93,500	85.00	67,375	61.25
3.	Imit. blood orange flavour, (Fir)	0.01	88,125	80.11	66,250	60.23
4.	Essence peach blossom, (RD)	0.01	76,250	69.32	67,375	61.25
5.	Oil sweet orange sesquiterpeneless, (Fr)	0.01	99,250	90.23	69,500	63.18
	Imit. blood orange flavour, (Fir)	0.01	...	...	...	...
	Essence peach blossom, (RD)	0.01	...	...	...	...
6.	Fritzbro Superarome imit. raspberry, (Fr)	0.10	101,875	92.61	73,500	66.82
	Essence peach blossom, (RD)	0.01	...	...	...	...
7.	Imit. cherry flavor, (MMR)	1.50	75,750	68.86	NI[b]	...
8.	Magna imit. honey flavor, (MMR)	0.80	70,875	64.43	NI[b]	...
9.	Imit. maple flavour, (Fir)	0.75	75,125	68.30	NI[b]	...

[a] Preparations contained a 10% excess of penicillin.
[b] No inhibition.

TABLE IV.—RESULTS OF ACCELERATED PENICILLIN STABILITY TESTS ON 200,000-UNIT[a] PENICILLIN-TRIPLE SULFONAMIDE POWDER

	Flavor	% Conc. of Flavor	Units/2.5 Gm. Powder Initial Assay	% of Theoretical	Heated 4 Days at 100°	% of Theoretical
Series I						
1.	Unflavored	....	224,000	101.82	134,000	60.91
2.	Astoria flavor No. 25, (DO)	0.10	208,000	94.55	<50,000	<22.73
3.	Arome betel, (Fir)	0.10	216,000	98.18	<50,000	<22.73
4.	Vanillin, U. S. P.	0.50 w/v	248,000	112.73	<50,000	<22.73
5.	Coumarin, N. F.	0.04 w/v	242,000	110.00	<50,000	<22.73
6.	Palatone, (Dow)	0.02 w/v	246,000	111.82	<50,000	<22.73
7.	Cyclotene, (Dow)	0.02 w/v	224,000	101.82	<50,000	<22.73
8.	Vanillin, U. S. P.	0.50 w/v	250,000	113.64	<50,000	<22.73
	Coumarin, N.F.	0.04 w/v	....	....	....	....
	Palatone, (Dow)	0.02 w/v	....	....	....	....
	Cyclotene, (Dow)	0.02 w/v	....	....	....	....
9.	Fritzbro Arome strawberry imit., (Fr)	1.0	216,000	98.18	<50,000	<22.73
10.	Cosmo N. A. flavor imit. root beer, creamy, (DO)	0.20	226,000	102.73	<50,000	<22.73
Series II						
11.	Unflavored	....	202,000	91.82	176,000	80.00
12.	Essence Fritzbro lime conc., (Fr)	0.05	195,000	88.64	180,000	81.82
13.	Ekomo flavor imit. raspberry, (Fr)	0.10	195,000	88.64	172,000	78.18
14.	Oil marshmallow flavor, (Fr)	0.10	204,000	92.73	168,000	76.36
15.	Essence Fritzbro orange conc., (Fr)	0.10	196,000	89.09	174,000	79.09
16.	Oil sweet orange sesquiterpeneless, (Fr)	0.025	208,000	94.55	132,000	60.00
	Essence peach blossom, (RD)	0.025	....	....	....	....
	Imit. blood orange flavour, (Fir)	0.025	....	....	....	....
17.	Anise oil, U. S. P.	0.10	196,000	89.09	168,000	76.36
18.	Clove oil, U. S. P.	0.10	183,000	83.18	164,000	74.55
19.	Soft drink powder flavor root beer, (Fr)	0.30	194,000	88.18	168,000	76.36
20.	Magna imit. maple flavor extra, (MMR)	0.05	190,000	86.36	182,000	82.73

[a] Preparations contained a 10% excess of penicillin.

Fig. 2. The three powders were also subjected to an accelerated penicillin stability test of four days at 100°, and these results are indicated by an asterisk on each graph.

Stability of Restored Suspensions.—Two unflavored controls, two 100,000-unit, and one 250,000-unit flavored penicillin-triple sulfonamide powders were restored with the proper amount of water and assayed for penicillin initially and following one week's storage at 5°. The results of these penicillin stability tests are shown in Table VI.

DISCUSSION

Only flavors that did not cause decomposition of penicillin could be used in these preparations. Penicillin salts have been known to be inactivated by many reagents such as acids, alkalies, oxidizing agents, glycerin, and many alcohols. Reaction

TABLE V.—RESULTS OF ACCELERATED PENICILLIN STABILITY TESTS ON 250,000-UNIT[a] PENICILLIN-TRIPLE SULFONAMIDE POWDER

Flavor	% Conc. of Flavor	Units/2.5 Gm. Powder: Initial Assay	% of Theoretical	Heated 4 Days at 100°:	% of Theoretical
1. Unflavored	...	282,500	94.17	240,000	80.00
2. Essence Fritzbro lime conc., (Fr)	0.1	280,000	93.33	233,750	77.92
3. Ekomo flavor imit. raspberry, (Fr)	0.1	275,000	91.67	245,000	81.67
4. Oil marshmallow flavor, (Fr)	0.2	272,500	90.83	245,000	81.67
5. Essence Fritzbro orange conc., (Fr)	0.1	267,500	89.17	240,000	80.00
6. Oil sweet orange sesquiterpeneless, (Fr)	0.025	270,000	90.00	255,000	85.00
Essence peach blossom, (RD)	0.025	...	...	...	...
Imit. blood orange flavour, (Fir)	0.025	...	...	...	...
7. Anise oil, U. S. P.	0.1	271,250	90.42	248,750	82.92
8. Clove oil, U. S. P.	0.1	275,000	91.67	253,750	84.58
9. Soft drink powder flavor root beer, (Fr)	0.3	271,250	90.42	237,500	79.17
10. Magna imit. maple flavor extra, (MMR)	0.5	265,000	88.33	253,750	84.58

[a] Preparations contained a 20% excess of penicillin.

TABLE VI.—PENICILLIN STABILITY IN PENICILLIN-TRIPLE SULFONAMIDE SUSPENSIONS

Flavor	% Conc. of Flavor	Lot No.	Units/5 ml. Suspension: Initial Assay	% of Theoretical	1 Week 5°:	% of Theoretical
Unflavored[a]	..	1	99,400	73.63	125,000	92.59
Unflavored[a]	..	2	126,500	93.70	104,000	77.04
Oil sweet orange sesquiterpeneless,[a] (Fr)	0.01	3	118,750	87.96	127,000	94.07
Essence peach blossom, (RD)	0.01		...	...	...	...
Imit. blood orange flavour, (Fir)	0.01		...	...	...	...
Oil sweet orange sesquiterpeneless,[a] (Fr)	0.01	4	132,125	97.87	111,000	82.22
Essence peach blossom, (RD)	0.01		...	...	...	...
Imit. blood orange flavour, (Fir)	0.01		...	...	...	...
Anise Oil,[b] U. S. P.	0.10	5	299,400	99.80	268,500	89.50

[a] Preparations contained 100,000 units plus a 35% excess of penicillin per 2.5 Gm. powder.
[b] Preparation contained 250,000 units plus a 20% excess of penicillin per 2.5 Gm. powder.

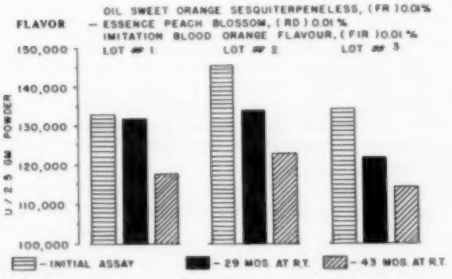

Fig. 1.—Results of long-term penicillin stability tests on 100,000 unit penicillin-triple sulfonamide powder. Preparations contained a 35% excess of penicillin and 0.25 Gm. total sulfonamides. The same flavor mixture was used in each lot.

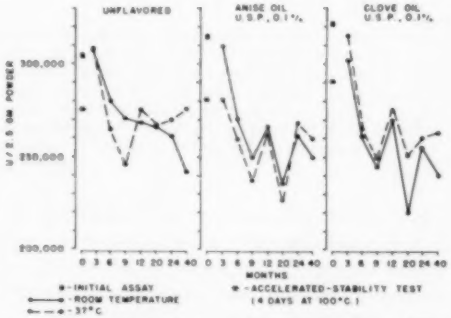

Fig. 2—Results of long-term penicillin stability tests on 250,000 unit penicillin-triple sulfonamide powder. Preparations contained a 20% excess of penicillin.

with these reagents, however, depends entirely upon the individual compound. Furthermore, since the structure of most of the flavoring agents or composition of the vehicles was not known, it was necessary to conduct stability studies. In some cases the chemical structure was known and it was possible to make a prediction of stability, but as we found, this method was not dependable. Vanillin, an aldehyde, and coumarin, a lactone, caused practically complete inactivation of penicillin in the accelerated test. On the other hand, clove oil, largely a phenol, and anise oil, essentially an ether, proved to cause little decomposition of the penicillin. The need for testing each flavoring agent individually is, therefore, quite evident.

The accelerated stability test served as a rapid means of eliminating those flavors that caused excessive destruction of penicillin activity. To reduce the number of samples, the flavors subjected to the accelerated test were those selected by a panel on the basis of taste. The basis of the accelerated test in the prediction of penicillin stability was the loss in activity after heating at 100° for four days. Although losses greater than 10% in activity are

usually an indication of instability of the penicillin (5), one flavor combination consisting of oil sweet orange sesquiterpeneless (Fr), essence peach blossom (RD), and imitation blood orange flavour (Fir) was so well preferred by the panel on the basis of taste that it was used for long-term stability studies in spite of the 27% penicillin loss in the accelerated test.

The results of these studies have shown that the penicillin in our flavored preparations loses very little activity and retains the labeled claim for as long as forty-three months. The accelerated stability test appears to be a useful tool, but not the final criterion in predicting the shelf stability of penicillin in preparations of this type. Although we have not included a report of the stability of the sulfonamides in these mixtures, we have found them to be stable under these conditions.

SUMMARY

1. A number of flavors have been screened for their ability to mask the bitterness of 100,000-unit, 200,000-unit, and 250,000-unit penicillin-triple sulfonamide powders.

2. An accelerated stability test has been used as an aid in determining those flavors that did not cause complete or excessive destruction of penicillin activity.

3. The penicillin in several flavored penicillin-triple sulfonamide powders has been found to be within label claim following forty-three months' storage at room temperature.

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Adsorption Studies on Clays I.*

The Adsorption of Two Alkaloids by Activated Attapulgite, Halloysite, and Kaolin

By MARTIN BARR† and EDWARD S. ARNISTA‡

Adsorption studies on strychnine and atropine by activated attapulgite, halloysite, and kaolin have been carried out. The activated attapulgite and halloysite were found to be superior adsorbents to kaolin. The adsorptive properties of the clays were not reduced after they were washed with acid and gastric juice. Activated attapulgite and halloysite had superior adsorptive properties at pH 6.8 and 7.2 than at higher hydrogen-ion concentrations. The results indicate that activated attapulgite and halloysite warrant further investigations as ingredients in intestinal adsorbent preparations.

A PRELIMINARY STUDY in our laboratory (1) revealed that the clays attapulgite and halloysite are superior to kaolin as adsorbents for strychnine, quinine, and atropine. It occurred to us that, since these clays were superior to kaolin in adsorbing alkaloids, they may also be superior in adsorbing toxins and bacteria. If investigations establish these facts, then halloysite and attapulgite should find a definite place in the formulation of intestinal adsorbent preparations. With these thoughts in mind, a series of studies was undertaken in order to establish definitely the relative adsorptive capacities of kaolin, and commercially available halloysite, and attapulgite for alkaloids, bacteria, and toxin.

This paper deals with the results of the study of the adsorptive properties of the clays for alkaloids. The comparative adsorptive capacities of the clays for bacteria and toxin is reported in two additional papers of this series.

EXPERIMENTAL

Materials.—The attapulgite and halloysite, which were investigated in our preliminary study, were obtained[1] in rock-like pieces and finely powdered before investigation. It was decided that, since one or both of the clays may reveal properties which would lead to their use in intestinal adsorbent formulations, commercially-available sources of the clays should be sought and clays from these sources should be studied in these investigations.

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This research was supported by a grant from the Wyeth Laboratories, Radnor, Pa.
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[1] Ward Natural Science Establishment, Rochester, N. Y.

A source[a] for halloysite was decided upon. The commercially available clay is off-white in color and is available in a powdered form having about a 60% minimum 0–20 μ in particle size and a 95% minimum which passes a 200-mesh sieve.

The attapulgite decided upon was an activated attapulgite[b] which has its adsorptive properties increased as a result of heating. It is a uniform, ultrafine, light-cream colored product. From 90 to 95% of the clay is finer than 10 μ and all of it passes through a 325-mesh sieve. A typical chemical analysis of the activated attapulgite is presented in Table I.

TABLE I.—TYPICAL CHEMICAL COMPOSITION OF HALLOYSITE AND ACTIVATED ATTAPULGITE

	Halloysite,[a] %	Activated Attapulgite,[b] %
SiO_2	56.2	67.0
Al_2O_3	40.3	12.5
Fe_2O_3	0.5	4.0
MgO		11.0
CaO		2.5
	1.2	
P_2O_5		...
TiO_2		0.5
K_2O	...	0.6
SO_3	1.8	...
Misc.	...	1.9
Total	100.0	100.0

[a] Supplied by Filtrol Corporation, Los Angeles, Calif.
[b] Supplied by Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa. Its chemical composition is presented on a volatile free basis.

Several kaolins were investigated and one[c] which appeared slightly superior in adsorptive properties was selected for these studies.

Adsorption studies on activated attapulgite, halloysite, and kaolin for strychnine and quinine were carried out at room temperature (approximately 24°) in the manner previously described (1). Isotherms were obtained for the untreated clays and for the acid-washed and gastric juice-washed clays in unbuffered suspensions and in suspensions buffered[d] at pH 6.8 and 7.2.

Preparation of Acid-Washed and Gastric Juice-Washed Clays.—Twenty grams of each clay were shaken with 1000 ml. of 0.05 *N* HCl or 1000 ml. of simulated gastric fluid T. S., U. S. P. XV (2) for a period of four hours in a mechanical shaker. The clays were then separated by filtration and washed once with purified water. They were then dried at 90° in an oven and were finely powdered.

Calculation of the Adsorptive Capacities of the Clays.—The method used for the calculation of the adsorptive capacities of the clays has previously been defined (1). In brief, application is made of the Langmuir equation which in linear form is:

[a] Filtrol Corporation, Los Angeles, Calif.
[b] Attasorb, Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa. This clay is now obtainable as Attasorb LVM, Minerals and Chemicals Corporation of America, Menlo Park, N. J.
[c] Kaolin, N. F. X., Merck and Company, Rahway, N. J.
[d] Buffer solutions were prepared by mixing together various proportions of 0.015 *M* sodium acid phosphate and 0.015 *M* sodium phosphate solutions. The molarity was kept low in order to minimize the interference of alkaloidal adsorption.

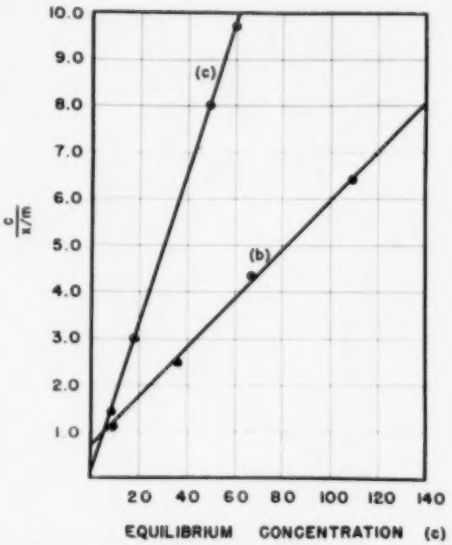

Fig. 1.—Linear form of the adsorption isotherms at 24° C. for strychnine by clays in unbuffered suspensions: (b) halloysite; (c) kaolin. It was not possible to determine an isotherm for (a) attapulgite as the free alkaloid precipitates due to the pH of the suspension.

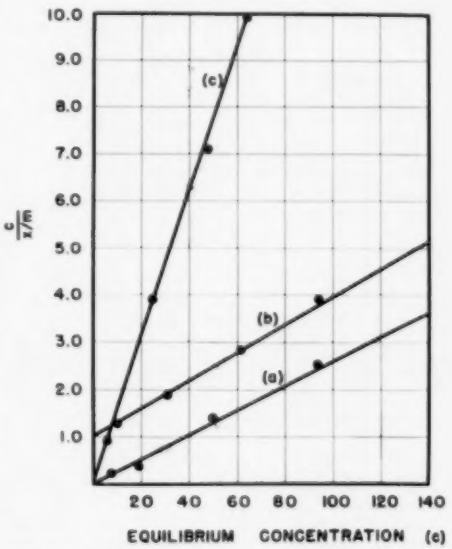

Fig. 2.—Linear form of the adsorption isotherms at 24° C. for strychnine by clays in suspensions buffered at pH 6.8: (a) activated attapulgite; (b) halloysite; (c) kaolin.

$c/(x/m) = (1/ab) + (c/b)$, where x/m = mg. alkaloid base adsorbed per Gm. of adsorbent, c = equilibrium concentration in mg. alkaloid base/100 ml., and a and b are constants. By plotting $c/(x/m)$ against c, linear curves are obtained from which may

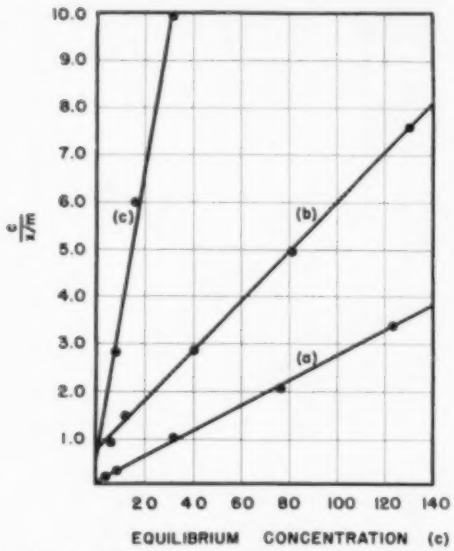

Fig. 3.—Linear form of the adsorption isotherms at 24° C. for strychnine by acid-washed clays in unbuffered suspensions: (a) activated attapulgite; (b) halloysite; (c) kaolin.

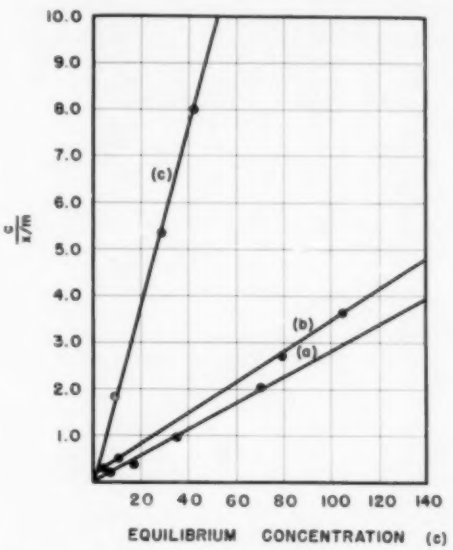

Fig. 4.—Linear form of the adsorption isotherms at 24° C. for strychnine by acid-washed clays in suspensions buffered at pH 6.8: (a) activated attapulgite; (b) halloysite; (c) kaolin.

TABLE II.—b VALUES OF CLAYS FOR STRYCHNINE[a]

	Activated Attapulgite	Halloysite	Kaolin
Unmodified Clay			
Unbuffered	[b]	19.1(3.9)	6.2(5.1)
Buffered (6.8)	38.7	32.8	6.6
Acid-Washed Clay			
Unbuffered	37.2(3.6)	18.5(3.8)	4.5(4.5)
Buffered (6.8)	37.9	31.0	5.3
Buffered (7.2)	[c]	[c]	[c]
Gastric Juice-Washed Clay			
Unbuffered	38.9(5.5)	24.4(3.9)	6.9(4.8)
Buffered (6.8)	41.4	35.3	6.1
Buffered (7.2)	[c]	[c]	[c]

[a] Numbers within parentheses represent pH values of suspensions.
[b] The adsorptive capacity cannot be determined as the alkaloidal base precipitates at the pH of the suspension (9.0).
[c] The adsorptive capacities cannot be determined in suspensions buffered at pH 7.2 since the alkaloidal base precipitates at this pH.

TABLE III.—b VALUES OF CLAYS FOR QUININE[a]

	Activated Attapulgite	Halloysite	Kaolin
Unmodified Clay			
Unbuffered	[b]	19.0(3.9)	4.7(5.1)
Buffered (6.8)	37.9	31.1	5.1
Acid-Washed Clay			
Unbuffered	34.3(5.6)	14.1(3.8)	3.5(4.5)
Buffered (6.8)	38.2	36.5	8.8
Buffered (7.2)	38.0	36.6	8.6
Gastric Juice-Washed Clay			
Unbuffered	31.2(5.5)	19.7(3.9)	5.1(4.8)
Buffered (6.8)	36.2	33.9	7.4
Buffered (7.2)	34.0	33.0	7.0

[a] Numbers within parentheses represent pH values of suspensions.
[b] The adsorptive capacity cannot be determined as the alkaloidal base precipitates at the pH of the suspension (9.0).

be found the slopes and *y*-intercepts. The closer the line is to paralleling *c*, the greater the adsorptive capacity of a clay. The constant *b* may then be calculated for each clay-alkaloid combination. The constant *b* is expressed in terms of mg. alkaloid base adsorbed per Gm. of adsorbent. The value of constant *b* is a good measure of the adsorptive capacity of adsorbents.

The linear forms of the adsorption isotherms at 24° for strychnine by the unmodified, acid-washed and gastric juice-washed clays in unbuffered and buffered suspensions are expressed in Figs. 1–6 incl. Isotherms representing the quinine-clay adsorptions are not presented because of space considerations and also because they closely parallel the strychnine-clay curves. The *b* values of the clays, as calculated from their linear curves, are listed in Tables II and III.

DISCUSSION

Examination of the data reveals that activated attapulgite is superior to both halloysite and kaolin as an adsorbent for strychnine and quinine, halloysite being superior to kaolin. It is apparent that activated attapulgite has approximately five to eight times the adsorptive capacity for the alkaloids as compared to kaolin while halloysite has about three to five times the capacity.

Activated attapulgite is superior to halloysite and kaolin as an adsorbent for strychnine and quinine, regardless of whether it is used as the original heat-treated clay, the acid-washed clay, or the gastric

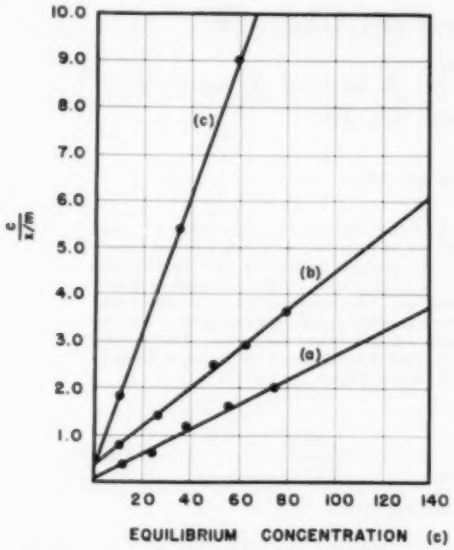

Fig. 5.—Linear form of the adsorption isotherms at 24° C. for strychnine by gastric juice-washed clays in unbuffered suspensions: (a) activated attapulgite; (b) halloysite; (c) kaolin.

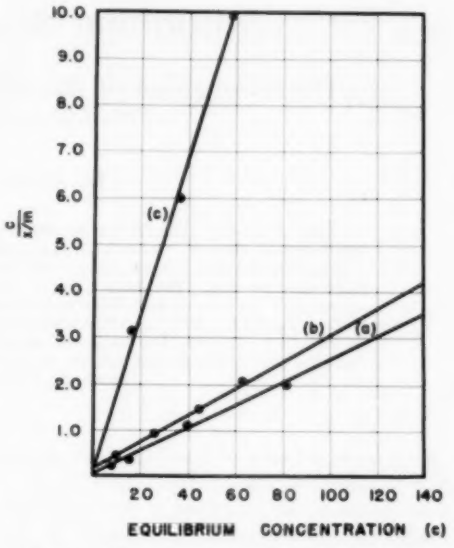

Fig. 6.—Linear form of the adsorption isotherms at 24° C. for strychnine by gastric juice-washed clays in suspensions buffered at pH 6.8: (a) activated attapulgite; (b) halloysite; (c) kaolin.

juice-washed clay; or whether the clay suspensions are buffered to pH 6.8, or 7.2, or unbuffered.

It was not possible to study the adsorption of the strychnine and quinine by the activated attapulgite in unbuffered suspensions, or of strychnine in suspensions buffered at pH 7.2, since the pH of these suspensions caused the precipitation of the free bases. It was observed that the acid-washed and gastric juice-washed clays had adsorptive capacities very similar to the original heat-treated clay and that these capacities were not significantly changed when the clays were studied in vehicles which were unbuffered or buffered at pH 6.8.

It was not possible to study the adsorption of strychnine by halloysite in suspensions buffered at pH 7.2 since the alkaloidal base precipitates at this pH. The commercially obtained halloysite and the acid-washed and gastric juice-washed halloysites have adsorptive capacities for the alkaloids which approximate each other in unbuffered suspensions (pH 3.8–3.9). It was noted, however, that at pH 6.8 and 7.2, their adsorptive capacities increased significantly and in a relatively uniform manner.

The *b* vaules obtained for the various forms of kaolin are so similar to each other that no distinguishing conclusions as to the effect of pH on its adsorptive capacity could be made.

The alkaloidal adsorption studies have revealed several significant facts which indicate that activated attapulgite and halloysite may serve effectively in intestinal adsorbent suspensions: they are more superior adsorbents for alkaloids than kaolin; their adsorptive capacities are not reduced by acid washing or gastric juice washing; and their adsorptive capacities are increased at pH 6.8 and 7.2 which approximate the pH of the intestinal fluids.

SUMMARY

1. The adsorptive capacities of activated attapulgite, halloysite, and kaolin for two alkaloids (strychnine and quinine) have been studied.

2. Activated attapulgite was found to be superior to halloysite and kaolin in adsorbing the alkaloids. This superiority was demonstrated with the original heat-treated clay, the acid-washed clay and the gastric juice-washed clay.

3. Activated attapulgite was found to be approximately five to eight times superior to kaolin as an adsorbent for alkaloids. Halloysite was found to be three to five times superior to kaolin as an alkaloidal adsorbent.

4. Acid washing and gastric juice washing did not reduce the adsorptive capacities of the clays for the alkaloids.

5. The adsorptive capacities of the activated attapulgite and halloysite were found to be increased significantly at pH 6.8 and 7.2.

6. The results indicate that activated attapulgite and halloysite warrant further investigation as ingredients in intestinal adsorbent preparations.

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Adsorption Studies on Clays II.*

The Adsorption of Bacteria by Activated Attapulgite, Halloysite, and Kaolin

By MARTIN BARR†

A method for the quantitative determination of the adsorption of bacteria by clays has been described. Studies on the adsorption of four bacteria (all known causative agents in enteric infections) by activated attapulgite, halloysite, and kaolin have been carried out. The four bacteria were *Staphylococcus aureus, Proteus vulgaris, Salmonella enteritides,* and *Shigella paradysenteriae.* Activated attapulgite was found to be superior to kaolin and halloysite as adsorbents for *Staphylococcus aureus.* None of the clays was found to be good adsorbents for the other bacteria. The results indicate that activated attapulgite warrants further investigation as an ingredient in intestinal adsorbent preparations.

APPLICATION has been made of kaolin as an ingredient in intestinal adsorbent preparations for use in the treatment of various enteric conditions. Clinically, it is thought to be effective in the treatment of those diarrheal states caused by an agent capable of being adsorbed, including those due to food poisoning and bacterial, or parasitic infections of the intestinal tract (1).

Since it was found in an earlier study (2) that activated attapulgite and halloysite are superior adsorbents for strychnine and quinine than kaolin, it was decided to compare the adsorptive properties of these clays for several bacteria known to be responsible for intestinal infections. This should throw further light on whether activated attapulgite and halloysite may be useful as ingredients of intestinal adsorbent preparations.

No mention has been made in the literature on the possible adsorption of bacteria by activated attapulgite and halloysite.

Numerous reports on the adsorption of bacteria by kaolin, some conflicting, have been published. Without attempting to completely review the literature, a few will be mentioned. Walker (3) rationalized that the usefulness of kaolin in the treatment of intestinal infections was due in some respect to adsorption of the bacteria. Eisler (4) reported that cholera vibrions were adsorbed by kaolin but typhoid bacilli were not. Gunnison and Marshall (5) found that kaolin had little capacity to adsorb *Escherichia coli, Clostridium welchii,* and *Lactobacillus acidophilus;* although it did adsorb *Staphylococcus aureus, Sarcina lutea,* and *Bacillus subtilis.* On the other hand, Smith (6) reported that a kaolin-aluminum hydroxide mixture was capable of adsorbing *Escherichia coli.*

EXPERIMENTAL

The sources of the activated attapulgite,[1] halloysite,[2] and kaolin[3] were similar to those used in the study of the adsorption of alkaloids (2).

Various techniques were investigated for their adaptability to the study of the adsorption of bacteria by the clays. The methods of Braafladt (7) and Eisenberg (8) were tried, but were not found to give reproducible results. A method was developed in our own laboratory which is reproducible within 10%.

Method for the Study of Bacterial Adsorption.—Five milliliters of an aqueous suspension of the microorganism under study were introduced into a sterile, capped, tube containing an accurately weighed quantity of clay which had been sterilized in the tube previously at 160° for two hours. The suspension was prepared by adding sterile purified water in small volumes to a bottle containing a twenty-four hour growth of the microorganism on veal agar. All clay-bacterial suspensions had a pH of 6.8 ± 0.1. The bacterial-clay suspension was then shaken[4] for thirty minutes. The tube containing the suspension was placed upright in a test tube rack and permitted to stand for three hours, during which time the clay settled to the bottom of the tube. The supernatant was then removed with a pipet and placed in another sterile tube and shaken in order to produce a uniform dispersion of the bacteria. A one-ml. aliquot of the supernatant was then removed with a pipet and a bacterial count made using the standard colony count procedure for the quantitative determination of microorganisms in water (9). Dilutions were so made that at no time were less than 30, nor more than 300, colonies counted

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This research was supported by a grant from the Wyeth Laboratories, Radnor, Pa.
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[1] Attasorb, Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa. This clay is now obtainable as Attasorb® LVM, Minerals and Chemicals Corporation of America, Menlo Park, N. J.
[2] Filtrol Corporation, Los Angeles, Calif.
[3] Merck and Company, Rahway, N. J.
[4] Variable Speed Shaking Apparatus, Arthur H. Thomas, Philadelphia, Pa.

TABLE I.—THE REDUCTION OF BACTERIA IN SUSPENSIONS BY ACTIVATED ATTAPULGITE

Mg. Clay in 5 ml. Bacterial Suspension	Bacteria	Bacteria in 5 ml. of Suspension	Bacteria in 5 ml. of Supernatant	Reduction in Bacteria
0	*S. aureus*	1.55×10^9	1.55×10^9	0
10	*S. aureus*	1.55×10^9	7.20×10^8	8.30×10^8
20	*S. aureus*	1.55×10^9	5.00×10^8	1.05×10^9
50	*S. aureus*	1.55×10^9	8.50×10^7	1.47×10^9
100	*S. aureus*	1.55×10^9	7.00×10^7	1.48×10^9
0	*P. vulgaris*	1.30×10^9	1.30×10^9	0
10	*P. vulgaris*	1.30×10^9	1.25×10^9	5.00×10^7
20	*P. vulgaris*	1.30×10^9	1.21×10^9	9.00×10^7
50	*P. vulgaris*	1.30×10^9	1.20×10^9	1.00×10^8
100	*P. vulgaris*	1.30×10^9	1.13×10^9	1.70×10^8
0	*S. enteritides*	2.03×10^9	2.03×10^9	0
10	*S. enteritides*	2.03×10^9	2.00×10^9	3.00×10^7
20	*S. enteritides*	2.03×10^9	1.95×10^9	8.00×10^7
50	*S. enteritides*	2.03×10^9	1.92×10^9	1.10×10^8
100	*S. enteritides*	2.03×10^9	1.90×10^9	1.30×10^8
0	*S. paradysenteriae*	1.66×10^9	1.66×10^9	0
10	*S. paradysenteriae*	1.66×10^9	1.65×10^9	1.00×10^7
20	*S. paradysenteriae*	1.66×10^9	1.62×10^9	4.00×10^7
50	*S. paradysenteriae*	1.66×10^9	1.60×10^9	6.00×10^7
100	*S. paradysenteriae*	1.66×10^9	1.56×10^9	1.00×10^8

TABLE II.—THE REDUCTION OF BACTERIA IN SUSPENSIONS BY ACTIVATED ATTAPULGITE, HALLOYSITE AND KAOLIN

Bacteria	Bacteria in 5 ml. Suspension	Bacteria in 5 ml. Supernatant after shaking Suspension with 10 mg. clay			Reduction in Bacteria		
		Activated Attapulgite	Halloysite	Kaolin	Activated Attapulgite	Halloysite	Kaolin
S. aureus	1.72×10^9	7.40×10^8	1.52×10^9	1.25×10^9	9.80×10^8	2.00×10^8	4.70×10^8
P. vulgaris	1.70×10^9	1.64×10^9	1.64×10^9	1.66×10^9	6.00×10^7	6.00×10^7	4.00×10^7
S. enteritides	2.03×10^9	2.00×10^9	2.01×10^9	2.01×10^9	3.00×10^7	2.00×10^7	2.00×10^7
S. paradysenteriae	2.17×10^9	2.15×10^9	2.15×10^9	2.13×10^9	2.00×10^7	2.00×10^7	3.00×10^7

in a single petri dish. The petri dishes were placed in an incubator at 37° and colony counts made after twenty-four hours. The number of bacteria adsorbed by the clay was the difference between the bacterial counts in the bacterial-clay supernatant and the control suspension which did not contain any clay.

In order to determine what concentration of clay should be used in the adsorption experiments, preliminary studies were undertaken. These studies revealed that activated attapulgite was the superior adsorbent of the clays. The reduction in count of four test bacteria by 10, 20, 50 and 100 mg. of activated attapulgite in 5 ml. of bacterial suspension was determined. The results are reported in Table I. It was found that, when activated attapulgite did reduce the bacterial count, 10 mg. of clay in 5 ml. of bacterial suspension brought about a sufficient reduction in bacterial count so that this clay-bacterial suspension ratio could be established as the basis for comparative experiments with halloysite and kaolin.

The bacteria included in these studies were *Staphylococcus aureus*,[5] *Proteus vulgaris*,[6] *Salmonella enteritides*,[7] and *Shigella paradysenteriae*.[8] All of these microorganisms are known causative agents in gastrointestinal tract infections.

The reduction in the number of bacteria in 5 ml. of the bacterial suspensions as affected by 10 mg. of activated attapulgite, halloysite, and kaolin are reported in Table II. All results listed in these tables represent the average of five determinations, all results being with a $\pm 10\%$ error.

The results in Table II indicate that whereas all three clays significantly reduce the bacterial count of the suspensions containing *Staphylococcus aureus*, there was only a small reduction in the count of the suspensions containing *Proteus vulgaris*, *Salmonella enteritides*, and *Shigella paradysenteriae*. Since we were concerned with the low amount of adsorption of the latter three bacteria by the clays, experiments were carried out in which the bacteria-clay ratios were changed. In one series, the number of bacteria in the suspensions, to which the clays were added, approximated the number which were found to be reduced by the clays in the initial studies (last three columns of Table II). A second determination was made for each clay-bacterial combination in which the number of bacteria in the suspensions approximated the number found to be reduced in the previous series. The reduction in bacterial count in the bacterial suspensions, after shaking with 10 mg. of activated attapulgite, halloysite, and kaolin, are reported in Table III.

[5] FDA No. 209, ATCC No. 6538.

[6] Isolated from a patient with pyelitis at the Hospital of the University of Pennsylvania, Philadelphia, Pa.

[7] Isolated from a patient with enteritis at Children's Hospital, Philadelphia, Pa.

[8] Isolated from a patient with bacillary dysentery by the Board of Health, Territory of Hawaii.

TABLE III.—THE DETERMINATION OF THE REDUCTION OF BACTERIA IN SUSPENSION BY CLAYS

Bacteria	Bacteria in 5 ml. Suspension	Bacteria in 5 ml. Supernatant after shaking Suspension with 10 mg. clay			Reduction in Bacteria		
		Activated Attapulgite	Halloysite	Kaolin	Activated Attapulgite	Halloysite	Kaolin
P. vulgaris	1.70×10^9	1.65×10^9	1.64×10^9	1.66×10^9	5.00×10^7	6.00×10^7	4.00×10^7
	3.92×10^7	3.91×10^7	3.62×10^7	3.84×10^7	1.00×10^6	3.00×10^6	8.00×10^5
	1.10×10^6	1.07×10^6	1.04×10^6	1.09×10^6	3.00×10^4	6.00×10^4	1.00×10^4
S. enteritides	2.03×10^9	2.00×10^9	2.01×10^9	2.01×10^9	3.00×10^7	2.00×10^7	2.00×10^7
	1.72×10^7	1.68×10^7	1.70×10^7	1.50×10^7	4.00×10^5	2.00×10^6	2.20×10^6
	2.10×10^6	2.05×10^5	2.04×10^5	2.08×10^5	5.00×10^3	6.00×10^3	2.00×10^3
S. paradysenteriae	2.17×10^9	2.16×10^9	2.15×10^9	2.13×10^9	1.00×10^7	2.00×10^7	4.00×10^7
	1.02×10^7	1.01×10^7	1.00×10^7	9.73×10^6	1.01×10^6	2.00×10^6	4.70×10^6
	1.13×10^6	1.11×10^5	1.09×10^5	1.10×10^6	2.00×10^3	7.80×10^3	3.00×10^3

DISCUSSION

It is apparent from the results in Table II that activated attapulgite, halloysite, and kaolin are capable of adsorbing *Staphylococcus aureus*. Under the test conditions, 10 mg. of activated attapulgite adsorbed nearly twice as many bacteria as did kaolin and about four times as many as halloysite. It is of interest to note that halloysite was found to be inferior to kaolin in adsorbing this bacterium.

It should be observed from Table I that whereas about 50% of the bacteria were removed from the *Staphylococcus aureus* suspension when it was shaken with 10 mg. of activated attapulgite, the amount of bacteria removed became greater as the concentration of clay was increased; over 95% of the bacteria was removed by 100 mg. of clay.

None of the clays appeared to adsorb *Proteus vulgaris*, *Salmonella enteritides*, and *Shigella paradysenteriae* to any great degree, as may be seen in Table II. In experiments in which the ratio of bacteria to clays was changed (Table III), results indicate that the adsorption of the bacteria by the clays was very small. Therefore, since the change of bacteria-clay ratios did not result in a significant increase in bacteria adsorbed, it is possible that these clays may not be capable of adsorbing the bacteria, but rather, the reduction in bacterial count may be due to the bacteria mechanically being brought down from suspension by the sedimenting clay particles.

It is not understood at this time why only *Staphylococcus aureus* was adsorbed by the clays. This bacterium is Gram-positive, whereas the three bacteria which were not adsorbed are Gram-negative. However, all four bacteria are negatively charged, as are the clays.

The pH under which the bacterial adsorption studies are carried out may also be an important factor. A change in hydrogen-ion concentration may alter the adsorption characteristics of the clay-bacterial suspensions by its influence on the electrical charge on the surface of the bacteria. The pH of the clay-bacterial suspensions in these experiments was 6.8 ± 0.1. This pH was due to the influence of the buffer salts of veal agar medium. The hydrogen-ion concentrations of all the suspensions were equal to each other, regardless of the clay, since the medium buffers resisted a change in pH by these substances. This pH was considered satisfactory for these experiments since it approximates the hydrogen-ion concentration of the intestinal tract.

The bacterial adsorption investigations have indicated that the clays studied are not satisfactory adsorbents for all bacteria. In the case of *Staphylococcus aureus*, which was adsorbed, activated attapulgite was found to be a superior adsorbent than kaolin and halloysite. It is understood that the four bacteria employed in this study are not the only causative microorganisms in enteric diarrheas, and in this respect, additional studies using other bacteria are indicated. Nevertheless, since activated attapulgite has been shown to be a superior adsorbent for *Staphylococcus aureus* and for alkaloids, there appears to be justification for its further investigations as an ingredient in intestinal adsorbent preparations.

SUMMARY

1. A method which makes possible the quantitative determination of the adsorption of bacteria by clays has been reported.

2. The adsorptive properties of activated attapulgite, halloysite, and kaolin for four bacteria have been studied.

3. Activated attapulgite, halloysite, and kaolin were found to be capable of adsorbing *Staphylococcus aureus*, but were not good adsorbents for *Proteus vulgaris*, *Salmonella enteritides*, and *Shigella paradysenteriae*.

4. Activated attapulgite was found to be superior to kaolin and halloysite in adsorbing *Staphylococcus aureus*.

5. The results indicate that activated attapulgite warrants further investigation as an ingredient in intestinal adsorbent preparations.

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Adsorption Studies on Clays III.*

The Adsorption of Diphtheria Toxin by Activated Attapulgites, Halloysite, and Kaolin

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A satisfactory method has been devised which makes it possible to determine the adsorptive capacities of clays for diphtheria toxin. Studies indicate that the activated attapulgites are superior adsorbents for the toxin than halloysite and kaolin. Activated attapulgite HVM was found to be the best adsorbent, seven times more effective than kaolin, and about twice that of halloysite. Studies on intestinal adsorbent suspensions indicate that the activated attapulgites should be investigated clinically as ingredients in these preparations.

KAOLIN HAS FOUND major use as an ingredient in the formulation of intestinal adsorbent preparations. Included in the rationale behind the use of kaolin in such preparations is the belief that the clay has adsorptive properties for toxins and bacteria, which are responsible for the onset of diarrhea, vomiting, nausea, and cramps in various types of intestinal infections (1–12).

The ability of kaolin to adsorb bacteria has been reviewed in an earlier paper (13). It has also been reported in this paper that kaolin has adsorptive properties for *Staphylococcus aureus*, but not for other bacteria.

There are many contradictory reports in the literature on the ability of kaolin to adsorb toxins. Some of these reports will be reviewed here.

Muller (14) working with mussel poison reported that kaolin had only limited adsorptive properties for the toxin. Also working with mussel poison, Swayne and Martin (15) concluded that kaolin was not an effective adsorbent. Moss and Martin (16) found that kaolin was ineffective in removing the toxic substances produced by *Eberthella typhosa*, *Shigella paradysenteriae*, or *Escherichia coli*. Eyre and Durch (17) found that kaolin possessed only slight ability to adsorb diphtheria toxin.

There have been many reports on the effectiveness of kaolin as an adsorbent for toxins. Mutch (18) found that kaolin adsorbed mussel poison, mushroom poison, and potato poison. The clay adsorbed toxins and toxic products of *V. cholerae*, *B. dysenteriae*, *B. enteritides*, *B. diphtheriae*, *B. botulinus*, *B. typhosus*, and *B. paratyphosus B* in studies carried out by Braafladt (19). Jordan (5), McRobert (6) and Schwartz (7) have reported on the successful use of kaolin in the treatment of patients suffering from bacterial food poisoning.

Kaolin has been utilized for its adsorptive properties, together with other substance, in many preparations intended for the treatment of enteric disturbances. Among these combinations are those with mineral oil (20), bismuth and tribasic calcium phosphate (21), bismuth subnitrate (8), calcium gluconate (9), calcium lactate (10), aluminum hydroxide (11, 22), a combination of mineral oil and aluminum hydroxide (23), and a mixture of pectin and aluminum hydroxide gel (24).

It has been reported in two earlier papers that activated attapulgite is superior to halloysite and kaolin as an adsorbent for strychnine and quinine (25) and *Staphylococcus aureus* (13). It was of interest to determine whether this relationship also exists for toxin. If experiments should reveal that activated attapulgite is a superior adsorbent for toxin, the clay would then be worthy of serious study as an ingredient in intestinal adsorbent preparations. This paper deals with such a study.

EXPERIMENTAL

Adsorption of Diphtheria Toxin by the Clays.—The sources of the activated attapulgite,[1] halloysite[2] and kaolin[3] were similar to those employed in the studies of the adsorption of alkaloids (25) and bacteria (13).

It became apparent upon reviewing the literature on the subject of the adsorption of toxins by clays that many of the contradictory results reported were probably due to the fact that the methods used in these studies were varied and left much to be desired. Consideration was given to the development of a toxin-adsorbing procedure in which

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[1] Attasorb, Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa. This clay is now obtainable as Attasorb® LVM, Minerals and Chemicals Corp. of America, Menlo Park, N. J.
[2] Filtrol Corporation, Los Angeles, Calif.
[3] Merck and Company, Rahway, N. J.

TABLE I.—ADSORPTION OF DIPHTHERIA TOXIN BY 0.1 GM. OF CLAYS

Clay	Lf Toxin Added per 50 ml.	Lf Toxin per 50 ml. at Equilibrium	Lf Toxin Adsorbed by 0.1 Gm. Clay
Activated Attapulgite	5250	4200	1050
Activated Attapulgite	4200	3150	1050
Activated Attapulgite	3150	2100	1050
Activated Attapulgite	2100	1050	1050
Halloysite	5250	4525	750
Halloysite	4200	3413	787
Halloysite	3150	2363	787
Halloysite	2100	1313	787
Kaolin	3150	2925	225
Kaolin	2625	2400	225
Kaolin	2100	1875	225
Kaolin	1575	1350	225

were determined and, from this, the total L_f units adsorbed by 0.1 Gm. of the clays was calculated. The results are expressed in Table I. The pH of each clay-toxin suspension was 6.7 ± 0.1.

In a second toxin adsorption experiment, varying quantities of the clays were added to 50 ml. of diphtheria toxin[4] (5250 L_f units) in 120-ml. wide-mouthed containers. The remainder of the procedure was similar to that just described. The pH of each clay-toxin suspension was 6.7 ± 0.1. The total L_f units of toxin adsorbed by the clays were calculated and appear in Table II.

The toxin adsorption studies were also carried out on acid-washed clays in a similar manner. The acid-washed clays were prepared as previously described (25). The pH of each acid-washed clay-toxin suspension was 6.5 ± 0.1, the buffer capacity of the toxin being sufficient to neutralize most of the acidity of the acid-washed clays. The L_f units

TABLE II.—ADSORPTION OF DIPHTHERIA TOXIN BY CLAYS

Clay	Gm. per 50 ml.	Lf Toxin Added per 50 ml.	Lf Toxin per 50 ml. at Equilibrium	Lf Toxin Adsorbed by Clay	Lf Toxin Adsorbed by 0.1 Gm. Clay
Activated Attapulgite	0.1	5250	4200	1050	1050
Activated Attapulgite	0.2	5250	3150	2100	1050
Activated Attapulgite	0.3	5250	2250	3000	1000
Activated Attapulgite	0.4	5250	1500	3750	939
Halloysite	0.1	5250	4463	787	787
Halloysite	0.2	5250	3625	1625	813
Halloysite	0.3	5250	2889	2361	778
Halloysite	0.4	5250	2138	3112	778
Kaolin	0.1	5250	5040	210	210
Kaolin	0.5	5250	4200	1050	210
Kaolin	1.0	5250	3150	2100	210
Kaolin	1.5	5250	2400	2850	187
Kaolin	2.0	5250	2025	3225	161

a quantitative evaluation of the adsorption of toxin by the clays under study could be made. After much thought, it was decided that a clay-toxin shakeout procedure should be used in which the amount of toxin adsorbed could be determined by titrating the toxin remaining in the supernatant with antitoxin, after the clay-toxin complex had been removed, by the use of a flocculation procedure. It therefore became necessary to select a toxin for which an antitoxin was available. It was for this reason that diphtheria toxin was decided upon for these toxin adsorption studies.

Method.—In the initial adsorption studies, 0.1 Gm. of the clays was added to varying toxin concentrations[4] contained in a finished volume of 50 ml. The mixtures were contained in 120-ml. wide-mouthed containers. They were shaken[5] for three hours and then centrifuged. (It was found that more uniform results could be obtained using a three-hour shaking period.) The clear supernatant liquids were collected and flocculation tests were performed on each supernatant using diphtheria antitoxin.[6] The L_f values per ml. of supernatant of toxin adsorbed by the acid-washed clays are reported in Table III.

Control flocculation determinations on the toxin itself revealed that the technique employed did not result in the deterioration of any toxin.

Comparative Adsorption Studies on Various Grades of Activated Attapulgite.—While the studies on toxin adsorption were being carried out, several new grades of activated attapulgite became available. These products represent heat-treated attapulgites which contain different amounts of volatile matter, namely water. These products are activated attapulgite LVM,[7] activated attapulgite RVM[8] and activated attapulgite HVM.[9]

Activated attapulgite LVM is a heat-treated attapulgite containing low volatile matter (26) and is similar to the activated attapulgite which has been used in studies on alkaloidal, bacterial, and toxin adsorption. Its ignition loss is 6% at 1800°F. Activated attapulgite RVM contains regular volatile matter (26), its ignition loss being 9% at 1800°F. Activated attapulgite HVM contains high volatile matter (26) and is a fine colloidal grade of activated attapulgite. Its ignition loss is 19% at 1800°F.

[4] Diphtheria toxin, Wyeth Laboratories, Lot 655, 105 L_f per ml. The various toxin concentrations were prepared by diluting the diphtheria toxin with sterile M/15 phosphate buffer solution, pH 6.8.

[5] Variable speed shaking apparatus, Arthur H. Thomas Co., Philadelphia, Pa.

[6] Diphtheria antitoxin, Wyeth Laboratories, Lot 121-1, 600 L_f per ml.

[7] Attasorb®, LVM, Minerals and Chemicals Corp. of America, Menlo Park, N. J.

[8] Attasorb®, RVM, Minerals and Chemicals Corp. of America, Menlo Park, N. J.

[9] Attasorb®, HVM, Minerals and Chemicals Corp. of America, Menlo Park, N. J.

TABLE III.—ADSORPTION OF DIPHTHERIA TOXIN BY ACID-WASHED CLAYS

Acid-washed Clay	Gm. per 50 ml.	Lf Toxin Added per 50 ml.	Lf Toxin per 50 ml. at Equilibrium	Lf Toxin Adsorbed by Clay	Lf Toxin Adsorbed by 0.1 Gm. Clay
Activated Attapulgite	0.1	5250	4200	1050	1050
Activated Attapulgite	0.2	5250	3375	1875	938
Activated Attapulgite	0.4	5250	1575	3675	919
Halloysite	0.1	5250	4500	750	750
Halloysite	0.2	5250	3600	1650	825
Halloysite	0.4	5250	3150	2100	788
Kaolin	0.2	5250	4875	375	188
Kaolin	0.6	5250	4200	1050	175
Kaolin	1.0	5250	3150	2100	210

Its average particle size is 0.32 μ, and it is lighter in color than the activated attapulgite used in these studies thus far.

Adsorption studies indicated that the HVM and RVM grades of activated attapulgite are superior to other grades as alkaloidal adsorbents. The LVM grade and the activated attapulgite are similar in their adsorptive capacities for alkaloids, as may be expected.

It was decided to compare the adsorptive capacities of the various grades of activated attapulgite for diphtheria toxin, both for the commercially-available clays and for the acid-washed products, using the procedure described earlier in this paper. The pH of each unwashed clay-toxin suspension was 6.7 ± 0.1; that of the acid-washed clay suspensions was 6.5 ± 0.1. The results of the adsorption of diphtheria toxin by 0.1 Gm. of these clays is reported in Table IV.

Adsorption of Diphtheria Toxin by Intestinal Adsorbent Preparations.—Since activated attapulgite has been shown to be superior to both halloysite and kaolin as an adsorbent for alkaloids (25) and *Staphylococcus aureus* (13), and since this study revealed it is a superior adsorbent for diphtheria toxin, it was decided to compare the toxin adsorption of several intestinal adsorbent preparations, in which the only difference in the formulas was the replacement of kaolin by the activated attapulgite. The HVM grade of activated attapulgite was selected for use in these preparations, since it was found to be superior to the other grades of activated attapulgite as an adsorbent in earlier experiments reported in this paper.

Preparations Studied.—Two widely used preparations, both finding application in the treatment of diarrheas, were selected for toxin adsorption studies in order to determine what effect the replacement of kaolin by activated attapulgite HVM had on this property. These preparations are identified as Intestinal Suspension A and B.[10] Intestinal Suspension A contains 10% kaolin and 36% of alumina gel (2% Al_2O_3) with flavoring, sweetening, coloring agents, and preservatives. Intestinal Suspension B contains 10% kaolin, 36% alumina gel (2% Al_2O_3), 0.9% pectin, and 5% heavy mineral oil with flavoring, sweetening, coloring agents, and preservatives. Suspensions were prepared similar in every respect to Intestinal Suspensions A and B except that the kaolin content was replaced by 10% w/v activated attapulgite HVM. Controls for each were also prepared, in which no kaolin or activated attapulgite HVM were present in the formulas. Studies on the adsorption of diphtheria toxin were carried out on each suspension.

[10] Wyeth Laboratories, Radnor, Pa.

TABLE IV.—ADSORPTION OF DIPHTHERIA TOXIN BY 0.1 GM. OF ACTIVATED ATTAPULGITES

Clay	Lf Toxin Adsorbed by 0.1 Gm. Clay
Activated Attapulgite[a]	1010
Acid-washed, Activated Attapulgite[a]	977
Activated Attapulgite LVM[b]	1025
Acid-washed, Activated Attapulgite LVM[b]	1025
Activated Attapulgite RVM[c]	1410
Acid-washed, Activated Attapulgite RVM[c]	1410
Activated Attapulgite HVM[d]	1575
Acid-washed Activated Attapulgite HVM[d]	1575

[a] Attasorb. [c] Attasorb RVM.
[b] Attasorb LVM. [d] Attasorb HVM.

TABLE V.—ADSORPTION OF DIPHTHERIA TOXIN BY INTESTINAL SUSPENSIONS CONTAINING ACTIVATED ATTAPULGITE HVM AND KAOLIN

Suspension	Clay	Lf Toxin Adsorbed by 1 ml. of Suspension
Intestinal Suspension A[a]	10% w/v Activated Attapulgite HVM[b]	1200
Intestinal Suspension A	10% w/v Kaolin	375
Intestinal Suspension A	none	225
Intestinal Suspension B[b]	10% w/v Activated Attapulgite HVM[b]	1500
Intestinal Suspension B	10% w/v Kaolin	250
Intestinal Suspension B	none	0–250

[a] Wyeth Laboratories, Radnor, Pa.
[b] Attasorb HVM.

Method.—A 2-ml. quantity of each suspension was added to 50 ml. of diphtheria toxin[4] (5250 L_f units) in 120-ml. wide-mouthed containers. They were shaken[5] for three hours and then centrifuged. The clear supernatant liquids were collected and flocculation tests were performed on each supernatant using diphtheria antitoxin.[6]

The L_f units of toxin per ml. of supernatant were

determined and from this, the total L_f units of toxin, adsorbed by 1 ml. of the suspensions, were calculated. Since the suspensions contain 10% w/v clay, one ml. of suspensions contains 0.1 Gm. of clay. The results are listed in Table V.

DISCUSSION

Results indicate that the method used for measuring the adsorption of diphtheria toxin by the clays is satisfactory. The method was found to be reproducible throughout the study and it permitted a quantitative measure of the adsorptive capacities of the clays for the toxin. Experiments have been carried out in an attempt to correlate adsorption of L_f units of diphtheria toxin by the clays with adsorption of minimum lethal doses (MLD).[1] It was found that there is a corresponding adsorption of MLD of diphtheria toxin equivalent to the L_f units of toxin adsorbed. This indicates that the toxin was adsorbed by the clays, as such, and was not being converted to toxoid during the adsorption procedure. It also indicates that an adsorption test procedure, such as the one utilized in this study, is a good measure of the reduction of the lethal activity of the toxin.

It may be observed from Table I that activated attapulgite has approximately five times the adsorptive capacity for diphtheria toxin as compared to kaolin; halloysite has about four times the capacity of kaolin.

An increase in the number of L_f units of toxin did not influence the number of units adsorbed by 0.1 Gm. of the clays; i.e., it was not possible to obtain an isotherm using 0.1 Gm. of clay.

One-tenth-gram quantities of activated attapulgite were found to adsorb about 1050 L_f units, similar quantities of halloysite adsorbed about 787 L_f units, and 0.1 Gm. quantities of kaolin adsorbed 225 L_f units of diphtheria toxin. Thus, the superiority of activated attapulgite and halloysite over kaolin for the adsorption of diphtheria toxin has been demonstrated.

Examination of Table II indicates that the amount of toxin adsorbed by 0.1 Gm. of clay is influenced to a degree when the total amount of clay is increased. The total amount of toxin adsorbed per 0.1 Gm. of clay was found to decrease slightly when the clay concentrations were increased. This is probably due to the fact that there is less total free-surface area available for adsorption upon increasing the amount of clay in the clay-toxin mixtures.

Once again, Table II reveals that activated attapulgite and halloysite are superior to kaolin in the adsorption of diphtheria toxin in the same relationship as previously found.

Examination of Table III reveals that there is no significant difference between the diphtheria toxin adsorbing properties of the clays, whether they are acid-washed or unwashed. The small differences noted in this table are within the experimental error of a flocculation procedure.

Comparative adsorption studies on the various grades of activated attapulgite (Table IV) indicate that activated attapulgite HVM is the superior adsorbent for diphtheria toxin. The superiority of this grade of activated attapulgite for diphtheria toxin is about one and one-half times that of the activated attapulgite used in previous studies and in the earlier parts of this study. Activated attapulgite HVM has approximately seven times the adsorptive capacity for diphtheria toxin as compared to kaolin and about twice that of halloysite.

Studies on the adsorptive capacity of intestinal adsorbent suspensions containing kaolin or activated attapulgite HVM reveal that the products containing the latter clay are superior in their ability to adsorb diphtheria toxin. The ratio of superiority is not quite as great as it is for the clays themselves, but it must be remembered that there are other ingredients in these suspensions, such as pectin and alumina gel, which could influence the degree of adsorption. The superiority of activated attapulgite HVM over kaolin as an adsorbent in these products is well defined, however.

The superiority of the activated attapulgites over kaolin and halloysite has been definitely established based on alkaloidal, bacterial, and toxin adsorption studies. Of these, its superiority as an adsorbent for diphtheria toxin is most striking. Since toxins are thought to be responsible for many of the ill-effects occasioned during an enteritis, there is good indication, based on the studies described, that activated attapulgites, and especially activated attapulgite HVM, are capable of serving as superior adsorbents in intestinal adsorbent preparations. The fact that these clays were found to be just as effective adsorbents after acid-washing is another indication for their usefulness as intestinal adsorbents. While it is understood that diphtheria toxin is not a causative agent in enteritis, it is very probable that the activated attapulgites are superior adsorbents for other toxins which are known to be responsible for such conditions. The lack of a ready available and stable enteric toxin and its antitoxin makes an *in vitro* study similar to that used in this investigation, very difficult. Clinical studies on the effectiveness of preparations containing activated attapulgites as adsorbents in the treatment of various forms of enteritis are necessary in order to finally establish the worth of these substances, as is the case with any new agent introduced for therapy.

SUMMARY

1. A satisfactory method has been introduced which makes possible a quantitative evaluation of the adsorptive properties of clays for diphtheria toxin.

2. It has been found that activated attapulgite has approximately five times the adsorptive capacity for diphtheria toxin as compared to kaolin, while halloysite has about four times the capacity.

3. Acid-washed activated attapulgite, halloysite, and kaolin were found to have similar adsorption properties for diphtheria toxin compared to the unwashed clays.

4. Activated attapulgite HVM has been found to be superior to activated attapulgite

[1] MLD studies carried out by Biological Laboratories of Wyeth Laboratories, Marietta, Pa.
The basis for the study was LD_{50} determinations on guinea pigs.

(LVM) in its adsorptive properties for alkaloids and diphtheria toxin.

5. Activated attapulgite HVM has been found to have seven times the adsorptive capacity for diphtheria toxin as compared to kaolin and about twice that of halloysite.

6. Two intestinal adsorbent suspensions containing kaolin were observed to have much greater adsorptive properties for diphtheria toxin when activated attapulgite HVM replaced kaolin in their formulas.

7. The fact that the activated attapulgites were found to be superior adsorbents to kaolin indicates that these substances should be investigated clinically as ingredients in intestinal adsorbent preparations.

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An Apparatus for Testing the Effect of Drugs on the Spontaneous Activity of the Rat*

By C. I. CHAPPEL, G. A. GRANT, S. ARCHIBALD, and R. PAQUETTE

A method is described for measuring the spontaneous activity of the rat. The apparatus consists essentially of a cage suspended by a cantilever beam of spring bronze. Movements of the animals are recorded by electrical counters connected to contacts on the spring assembly. The accuracy of this method and its application to pharmacological screening are discussed.

THE SCREENING of compounds for stimulant or sedative activity has been handicapped by lack of an easy, quick, and reproducible quantitative method for measurement of mildly depressed or stimulated states in experimental animals. This paper describes a method in routine use in this laboratory which has proved consistently reliable in the screening of over one hundred compounds.

In 1898, Stewart (1) described the first method for testing the effect of a drug on the activity of the rat. This technique which involved the use of a revolving drum was modified by Shirley (2) and Skinner (3). Tainter (4) used a similar apparatus in testing a series of analeptic drugs for stimulant activity. Other techniques have employed tambour-mounted cages (5, 6), spring-mounted cages with kymographic recording (7, 8), and photocells with electronic relays (9). Equally elegant methods using exercising turntables or tilt tables have also been suggested (10). It is not the purpose of this publication to discuss the merits or disadvantages of these methods in detail; for this, the reader is referred to a more comprehensive review on the subject (11). However, from a pharmacological standpoint the apparatus and methods are handicapped by lack of sensitivity, i. e., recording only gross body movements of the animals, difficulties in the quantitative evaluation of kymographic records, or features inherent in the apparatus which obviate its use in short-term studies.

APPARATUS AND GENERAL METHOD

This apparatus consists of a series of ten cages, one of which is illustrated in Fig. 1. The cages, which are 6 inches in diameter and $4\frac{1}{2}$ inches high, are made of 4×4 galvanized wire mesh, and suspended by means of $\frac{1}{4}$-inch diameter brass rod and

* Received February 25, 1957, from Ayerst, McKenna and Harrison, Ltd., Montreal, Canada.
The authors gratefully acknowledge the assistance of Hugh Green, B.A. Sc., in the construction of this apparatus.

TABLE I.—ANALYSIS OF VARIANCE

Nature of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio
Between months	4,778,457	2	2,389,229	3.21
Between days within months	729,655	2	364,828	<1
Between tests within days	662,719	1	662,719	<1
Between cages	3,660,556	9	406,728	<1
Error per rat	55,732,648	75	743,102	1
Total	65,564,035	89		

Error per rat $= \sqrt{743,102} = 862.$

Standard error for averages of 10 rats $= \frac{862}{\sqrt{10}} = 273.$

Grand averages count over the six month period = 1,613.
Precision = $(1,613 - (2 \times 273))/1,613 \times 100\% = 66\%$.

a ball and socket joint.[1] The suspension assemblies are brought to equal weight by addition of the appropriate amount of solder immediately below the ball and socket joint. The spring contact support for the cage is made of 0.050-in. phosphor bronze 15 in. × 1 1/16 in.; it is tapped 2 in. from one end to take the ball and socket joint and attached at the other end to a block of 1 3/4 in. wood which is 5 1/2 in. long and slopes from 3 5/8 in. to 2 1/4 in. The rigid contact support is an L-shaped brass bar 5/16 in. thick, 1 1/2 in. wide, and 9 in. long. It is tapped 1 in. from the end for a brass screw 2 in. long which is provided with a knurled hand wheel and a lock nut. Binding posts are supplied for the rigid and spring contacts. A pilot light with a NE-45 lamp is recessed in a wooden trough and mounted directly above the rigid bar contact. The pilot lights are not visible to animals in the cages. The electronic relays for these cages[2] and the counters[3] are mounted in a separate room to obviate the possibility of their sound interfering with the activity of the animals. The rigid and spring contacts of each cage are connected to the low-voltage probe circuit of the relay. The pilot lamp and electronic counter for each unit are connected to separate, normally open, contacts on the relay, so that when the primary circuit is closed by motion of the cage, both the lamp and counter circuits are activated.

All tests are done in a relatively sound proof room with constant illumination, and temperature controlled at 74° ± 1°F. The animals are maintained under these conditions for a period of twenty-four hours prior to testing.

Since female rats are subject to variations in spontaneous activity coincident with the various stages of their estrus cycle, only male animals are used (12–14). The albino strain maintained in this colony has been employed exclusively. The cages are designed for animals weighing between 150 and 170 Gm. In routine tests the animals are allowed full access to food and water until the test has begun, but neither is supplied to the animals in the test cage.

In the performance of routine tests a group of five animals are given the test drug and a similar group of control animals are given the vehicle alone. An interval of twenty minutes in the case of intraperitoneal or subcutaneous injection, or one hour after oral gavage, is allowed for absorption of the material. In some instances these intervals have been adjusted so that tests can be done during the period of peak activity of a more rapidly or more slowly absorbed compound. The animals are then placed individually in the cages. By means of the knurled screw the contacts are adjusted, while watching the pilot light, so that the degree of movement desired is being counted. At least ten animals are tested at each dose-level of the test compound and each group is compared to a similar number of control animals tested concurrently. The difference between the mean activity of the treated and control groups is expressed as a percentage of the control.

In order to achieve maximal sensitivity with this method, the contacts are adjusted so that any gross body movement, excluding that of respiration, is recorded. Thus movements are counted that are part of the cleaning habits of the rat, which are not recorded in devices such as the photo-cell apparatus

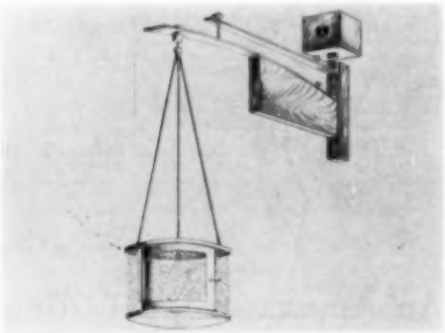

Fig. 1—Single activity cage.

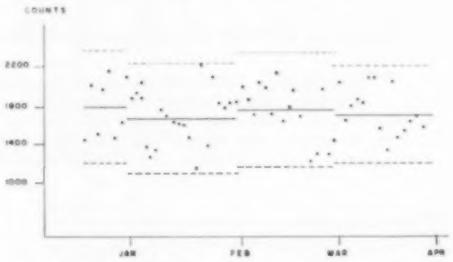

Fig. 2—Control studies. Each point represents the average number of counts of 20 animals. The solid lines are the monthly grand averages and the dotted lines the 95% confidence limits.

[1] Minneapolis Honeywell Ball and Socket Joint #27518.
[2] Electronic Relay Type 13DJ3, Electronics Corp. of America, Cambridge, Mass.
[3] Productimeter Model 6Y-1MF, Durant Mfg. Co., Milwaukee, Wis.

or tilt tables. The importance of these movements, as well as locomotion, in assessing drug activity has been emphasized by Chen (17). When the desired sensitivity has been found, the lock nut is then tightened and the movements of the animals recorded during the following forty-minute period.

RELIABILITY STUDY AND DISCUSSION

In Figs. 2 and 3 are shown the results of control studies using this apparatus over a three-and-a-half month period. Each point in Fig. 2 represents the mean number of activity counts for a group of 20 animals over a forty-minute period in one test day. The solid horizontal lines are the grand averages for each month and the dotted lines the 95%-confidence limits. Figure 3 is a histogram obtained from the same data. It may be seen that the distribution of the individual counts is normal, the index of symmetry of the curve being 0.14. A consideration of the sources of variation in this test may be found in Table I. The data used to make this analysis were obtained from 90 animals tested on alternate months over a six-month period from January to June. It may be seen that there is no significant variation introduced by the cages, nor is there a significant variation between tests run on the same day, or different days within a period of a month. A significant variation ($p = 0.05$) is seen between tests conducted in different months. (The F ratio in this analysis is 3.21, the critical value obtained from a table of F distribution is 3.12 for $p = 0.05$ (18).) This may reflect a seasonal variation in the activity of the rat which would have to be controlled in long-term studies. The overall precision of this method, as shown by the analysis of variance, is 66% with the greatest source of variation attributed to individual differences between animals.

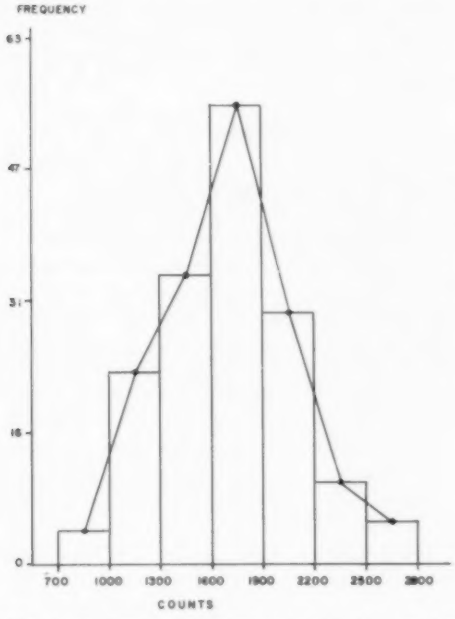

Fig. 3—Histogram obtained from the data of the three-month control study.

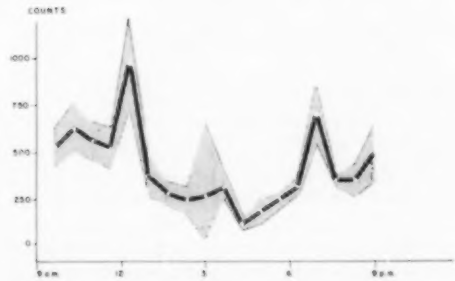

Fig. 4—Twelve-hour activity counts, each point represents the average accumulated count of 20 animals over a forty-minute period. The shaded area is the respective 95% confidence limits.

Figure 4 shows the mean activity counts recorded by a group of 20 animals maintained in the test cages over a twelve-hour period. The plotted points represent the accumulated counts over a forty-minute period, the dotted area is the 95%-confidence limits. The generally low activity of the rat during the afternoon is typical of this primarily nocturnal species. There also appears to be a cyclic activity rhythm of approximately three-hours' duration. Since these animals did not have access to food during this test, these cycles may reflect hunger contractions as demonstrated by Richter (5) and Shirley (2). Activity curves such as this have been used to study the duration of sedative or stimulant activity of synthetic compounds.

Examples of routine forty-minute tests for the potency of sedative or stimulant compounds are shown in Figs. 5 and 6. Amphetamine sulfate and

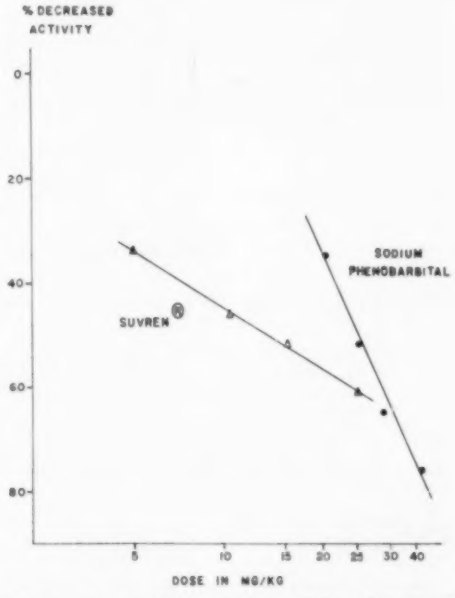

Fig. 5—Comparative sedative activity of Suvren® (Captodiamine hydrochloride) and phenobarbital.

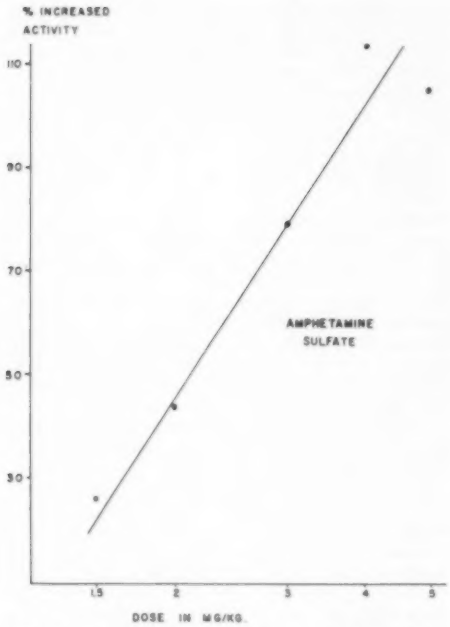

Fig. 6—Stimulant activity of amphetamine sulfate.

sodium phenobarbital were chosen as reference standards and their dose response curves after intraperitoneal administration are presented. Similar data are also shown for captodiamine hydrochloride (Suvren®), a potent, nonhypnotic, sedative antispasmodic described recently by Petersen (15).

Feurt and LaRocca (16), using a different electronic apparatus have found the mild sedative dose of pentobarbital to be 20 mg./Kg. after intraperitoneal injection in the mouse. The DDA_{50} of sodium phenobarbital, using this technique in the rat, is 25 mg./Kg. The rather flat dose-response curve for captodiamine has been found to be that expected of a sedative which differs from barbiturates, such as phenobarbital, in that at high dosage it is excitant and not hypnotic. Captodiamine exerts a considerable sedative effect at doses lower than those of phenobarbital, as shown by the reduction in spontaneous activity of the animals.

SUMMARY

A rapid and reliable method for screening compounds for sedative or stimulant properties which measures quantitatively changes in the spontaneous activity of test animals is described. Data are given from a reliability study which implicates individual differences between rats as the greatest source of variation. Dose-response curves using this method for three representative compounds: amphetamine sulfate, phenobarbital, and captodiamine hydrochloride are presented.

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Iodophors as Disinfectants*

By C. A. LAWRENCE, C. M. CARPENTER, and A. W. C. NAYLOR-FOOTE†

Comparative tests were made of the bactericidal and fungicidal activities of an iodophor (Wescodyne®), Roccal®, Amphyl®, and phenol. Roccal was the only germicide in the group that was adversely affected by a synthetic hard water solution. With the exception of phenol, the remaining germicides showed some loss in bactericidal activity when examined in the presence of serum. Wescodyne was found to be highly fungicidal and distinctly lethal for tubercle bacilli. The same compound was also effective in moderate concentrations against spores of *B. subtilis*.

ALTHOUGH THE CONVENTIONAL tincture and aqueous solutions of iodine are still the skin disinfectants of choice of many physicians, much of the decline in popularity has been the result of several undesirable properties of iodine preparations. These include the staining action on fabrics, the irritating effects on denuded tissues

* Received January 28, 1957, from Health Department, County of Los Angeles, Los Angeles 12, Calif.
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and mucus membranes, the relative insolubility in water, and the chemical sensitization of certain individuals to this halogen.

Recent studies (8,9) to develop new formulations containing iodine as the active germicidal agent have resulted in overcoming most of the undesirable features. In these new products, referred to as iodophors, elemental iodine is complexed with one or more nonionic wetting agents which act as solubilizers or carriers. Acids, such as phosphoric, are sometimes used to increase the iodine stability in the presence of organic soils (10). The bactericidal activity of iodine is enhanced in the iodophors and they retain their germicidal efficiency at comparatively low temperatures (11). The odor and staining tendencies are reduced to a minimal, and the solutions are practically free of any toxic or irritating properties (12).

The early use of iodine as a chemical agent for the destruction of microorganisms is probably antedated only by chlorine (1). While iodine was first used in medicine by Coindet (2) for the treatment of goiter, a tincture of iodine was used as early as 1839 by Davies (3) for the disinfection of wounds and a dilute solution of iodine was used in 1867 by Watson (4) in the treatment of gonorrhea. Davaine, in 1873, (5, 6) was first to demonstrate the sporicidal activity of iodine solutions upon the anthrax bacillus. A comprehensive review on iodine has been presented recently by Gershenfeld (7).

Lazarus (13), Johns (14), and Johns and Berzins (15) were the first to explore the use of iodophors as sanitizing agents for dairy farm use. Lazarus found one of these disinfectants would give desirable cleaning and sanitizing results on dairy utensils, rubber, milking machines, and on the udders of cows. Evidence was presented to indicate the superiority of an iodophor over that of chlorine in the destruction of *Salmonella typhosa*. Johns compared the germicidal activity of two iodophors with that of several chlorine and quaternary ammonium germicides. The iodophors were found to be as effective as the hypochlorites, and especially in the presence of 0.5 per cent of skim milk, when tested against *Micrococcus pyogenes*, var. *aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The other disinfectants were appreciably slower in their activity in corresponding tests. In a "capacity test" (16), the iodophors were found to be extremely active, frequently destroying many more increments of microorganisms than the other germicides at eight times greater concentration. Ortenzio (17) presented a tabulation of the results of a collaborative study on a commercial hypochlorite and an iodophor in which the latter was also found to be eight times more effective than chlorine (25 p.p.m. I_2 = 200 p.p.m. Cl_2, 12½ p.p.m. I_2 = 100 p.p.m. Cl_2; etc.). Johns also noted that the very marked inhibitory action of the iodophors against *Bacillus subtilis* spores could be attributed to the nonionic constituents in the formulations. This inhibitory action could be neutralized by 2 per cent skim milk in the culture media. Johns and Berzins (15) subsequently reported that the iodophors probably would not act as preservatives in milk at concentrations which would escape detection by taste.

Mueller (18) found that a liquid and a powdered iodophor compound were equally effective as germicidal agents against several microorganisms. Concentrations of 25 p.p.m. of available iodine in the iodophors compared favorably with 100 p.p.m. of available chlorine in destroying *S. typhosa*, *E. coli*, *M. pyogenes*, var. *aureus*, and *Ps. aeruginosa* in the presence of hard water (500 p.p.m. $CaCO_3$), 1 per cent whole milk, and 1 per cent dishwash soil. On the basis of these data, Mueller concluded, "According to these findings, the iodine detergent-sanitizers can be recommended for sanitizing food utensils, if field tests substantiate results of the laboratory performance tests."

Reference to the activity of an iodophor in the presence of hard water may also be found in an article by Terry and Shelanski (8). The compound was compared with two commercially available quaternaries in waters of 200 p.p.m. and 400 p.p.m. hardness. It was found that in water of 200 p.p.m. hardness, 50 p.p.m. of titratable iodine in the iodophor was equivalent to approximately 350 p.p.m. of one quaternary and 300 p.p.m. of the other one. In water of 400 p.p.m. hardness the 50 p.p.m. titratable iodine solution was equivalent to approximately 450 p.p.m. of one of the quaternaries and 375 p.p.m. of the other. The same authors noted that the iodophor was a better skin degerming agent than tincture of iodine and also better than a standard commercial germicidal detergent containing 3 per cent G-11 (hexachlorophene).

EXPERIMENTAL

Comparative Bactericidal Tests of Several Disinfectants in Distilled Water, Hard Water,[1] and

[1] A stock solution of hard water was prepared by adding 1.8 Gm. of $CaCl_2.2H_2O$ and 1.44 Gm. of $MgCl_2.6H_2O$ to one liter of distilled water. This provided a hardness, based upon calcium carbonate, of 2000 p. p. m. This stock solution was diluted 1:10 with distilled water to give 200 p. p. m. hardness and used as the diluting medium for the various disinfectants. A 10 per cent sterile horse serum solution in distilled water was also used to evaluate the germicides.

TABLE I.—A COMPARISON OF THE GERMICIDAL ACTIVITY OF SEVERAL DISINFECTANTS IN THE PRESENCE OF DISTILLED WATER, HARD WATER,[a] AND SERUM[b]

Organism	Highest Dilution Showing Activity in 10 Minutes at 20°											
	Wescodyne			Roccal			Amphyl			Phenol		
	Dist. H_2O	Hard H_2O	Serum	Dist. H_2O	Hard H_2O	Serum	Dist. H_2O	Hard H_2O	Serum	Dist. H_2O	Hard H_2O	Serum
M. pyogenes, var. *aureus*	(1:320)[c]	(1:640)	(1:80)									
	1:20,000	1:40,000	1:5,000	1:20,000	1:10,000	1:2,500	1:400	1:400	1:80	1:60	1:60	1:60
E. coli	(1:640)	(1:640)	(1:80)									
	1:40,000	1:40,000	1:5,000	1:20,000	1:10,000	1:2,500	1:800	1:800	1:80	1:80	1:80	1:80
S. typhosa	(1:640)	(1:1,280)	(1:160)									
	1:40,000	1:80,000	1:10,000	1:20,000	1:20,000	1:2,500	1:1,600	1:800	1:640	1:100	1:100	1:100
S. cholera suis	(1:640)	(1:640)	(1:160)									
	1:40,000	1:40,000	1:10,000	1:40,000	1:5,000	1:5,000	1:800	1:800	1:640	1:80	1:80	1:80
Ps. aeruginosa	(1:320)	(1:320)	(1:16)									
	1:20,000	1:20,000	1:1,000	1:10,000	1:2,500	1:1,000	1:200	1:200	1:80	1:80	1:80	1:80
Prot. vulgaris	(1:320)	(1:320)	(1:640)									
	1:20,000	1:20,000	1:40,000	1:10,000	1:2,500	1:1,000	1:1,600	1:1,600	1:640	1:100	1:100	1:100

[a] See footnote 1 for formula of the synthetic "Hard Water."
[b] Ten per cent horse serum in distilled water.
[c] Figures in parentheses indicate dilutions prepared of Wescodyne as taken from the original container. Figures below same parentheses indicate calculated dilutions based upon iodine present in solution. Figures under Roccal and phenol are dilutions based upon 100% active materials. Figures under Amphyl are dilutions based upon the germicide as taken from the original container.

Serum.—The activity of an iodophor[2] was compared with that of three commonly used disinfectants, Roccal,[3] Amphyl,[4] and phenol. The organisms used included one strain each of *M. pyogenes*, var. *aureus*, *E. coli*, *S. typhosa*, *Salmonella cholera suis*, *Ps. aeruginosa*, and *Proteus vulgaris*.

To 0.5 ml. of disinfectant solution was added 0.5 ml. of a twenty-four-hour broth culture of test organisms grown in beef extract broth. At the end of ten minutes and at a temperature of 20°, a 4-mm. loopful of the test suspension was transferred to a tube containing approximately 10 ml. of nutrient medium. Brewers' fluid thioglycollate medium was used in subculturing from Wescodyne and Roccal.[5] Plain beef extract broth was used as the subculture medium from Amphyl and phenol. Incubation of the subculture tubes was carried out at 37° for seven days and tubes showing no growth at this time were checked for bacteriostasis by transferring three 4-mm. loopfuls of the medium to additional tubes of the respective media. The results of this study are presented in Table I.

Examination of the data given in Table I will reveal the following pertinent points. On the basis of the active ingredient in Wescodyne (iodine) and Roccal (benzalkonium chloride U. S. P.), there is a suggestion that the former germicide is superior in activity to the quaternary ammonium compound. This is decidedly so in the study where hard water[6] is used as the diluting medium. In no instance were there any adverse effects noted of hard water upon the germicidal effects of Wescodyne. The germicidal activity of Roccal, on the other hand, was reduced by one to three increments upon dilution with hard water.

In comparing the activity of Wescodyne with Amphyl in the presence of distilled water and hard water, but here evaluating their performance on the solutions as derived from the commercial containers, there appears to be a reasonably close correlation in the germicidal activity of the two products. With the exception of phenol, all of the disinfectants showed an appreciable drop in activity when the tests were carried out in the presence of 10% horse serum.

Comparative Fungicidal Test of Several Disinfectants.—The fungicidal activity of Wescodyne was compared to the activity of Roccal, Amphyl, and phenol on several strains of pathogenic fungi. The organisms were grown for a period of four weeks at room temperature (24° to 26°), in 4-ounce screw-capped bottles containing glass beads and a medium of the following composition: glucose, 4 Gm.; Neopeptone, 1 Gm.; distilled water, 100 ml.;

[2] Wescodyne is a West Disinfecting Company, Long Island City, New York, brand of iodophor, containing "Active Ingredients: polyethoxy polypropoxy ethanol-iodine complex—7.75 per cent; nonyl phenyl ether of polyethylene glycol-iodine complex—7.75 per cent; hydrogen chloride—0.10 per cent (provides 1.6 per cent available iodine). Inert Ingredients: 84.4 per cent."

[3] Winthrop Laboratories, New York, brand of benzalkonium chloride, U. S. P.

[4] Lehn and Fink Products Corporation, New York, "Contains potassi m ricinoleate, O-phenyl-phenol, *p*-tert. amylphenol and S. D. Alcohol. Inert matter: water—16.0 per cent; propylene glycol—10.0 per cent; glycerol—4.0 per cent. Total inert matter—30.0 per cent; S.D. alcohol—6.2 per cent (by volume)."

[5] Three per cent Tween 20 and 0.2% Asolectin added to medium to inactivate quaternary.

[6] See footnote 1.

TABLE II.—A COMPARISON OF THE SPORICIDAL ACTIVITY OF SEVERAL DISINFECTANTS UPON PATHOGENIC FUNGI

Organism	Highest Dilution Showing Activity in 10 Minutes at 20°			
	Wescodyne	Roccal	Amphyl	Phenol
Microsporum audouini	(1:480)[a] 1:30,000	1:2,500	1:640	1:80
Microsporum gypseum	(1:320) 1:20,000	1:500	1:320	1:80
Tricophyton mentagrophytes	(1:400) 1:25,000	1:500	1:320	1:80
Tricophyton rubrum	(1:160) 1:10,000	1:100	1:20	1:80
Tricophyton tonsurans	(1:320) 1:20,000	1:400	1:160	1:40

[a] See legend *c* footnote Table I.

pH, 6.0. At the end of the incubation period, the heavy mycelial and spore mass was shaken vigorously with the glass beads and the suspension filtered through several layers of sterile gauze. Five-tenths milliliter of the suspension was added to 5 ml. of the disinfectant dilution. At the end of 10 minutes at 20°, a 4-mm. loopful of the test suspension was transferred to approximately 10 ml. of Neopeptone medium. The subculture tubes were incubated for one month at room temperature and tubes showing no growth at this time were checked for fungistatic activity by subculturing three loopfuls of the medium to a second tube containing broth. The latter was again incubated at room temperature for several weeks and the presence or absence of growth recorded. The results of this study are presented in Table II.

In comparing the fungicidal activity of Wescodyne and Roccal, and basing the effective killing concentration on the active ingredients of these disinfectants (titratable iodine and benzalkonium chloride, respectively), it may be noted that the iodophor is a far more superior fungicidal agent than the quaternary ammonium compound. On the basis of the disinfectant concentrations and dilutions made from the solutions from the commercial containers, there appears to be a close correlation in the fungicidal activity between Wescodyne and Amphyl. In general, phenol appears to show the same order of activity against fungi as against bacteria (Table I).

Germicidal Activity of Wescodyne on Tubercle Bacilli.—Sputum from a patient with untreated tuberculosis was digested with sodium hydroxide and inoculated on tubes of Löwenstein-Jensen medium and the latter incubated at 37° for two months. To a tared bottle containing glass beads was added 50 ml. of the moist culture and 5 ml. of sterile distilled water. The organisms were dispersed by shaking vigorously in a shaking machine for one hour.

To sterile 1-ounce screw-capped bottles were added 5-ml. amounts of diluted Wescodyne. One-milliliter amounts of the culture suspension were distributed in the diluted germicide. At the end of two minutes, to one series of culture-germicide suspension was added 5 ml. of sterile 0.003 *M* sodium thiosulfate solution to neutralize or inactivate the germicidal effects of the iodine. Another series of test suspensions was inactivated after the germicide had been allowed to act on the organisms for ten minutes.

One-tenth milliliter amounts of each test suspension, as well as a distilled water suspension of organisms without Wescodyne (control), were inoculated onto the surfaces of Löwenstein-Jensen medium and Herrold's medium. The same amount of material was also added to a tube of Dubos Broth Base Serum medium. The tubes were incubated at 37° for two months and the presence or absence of colonies on the medium recorded.

In addition to inoculating the culture suspensions on laboratory media, 1 ml. of each dilution suspension and untreated control was injected subcutaneously in the inguinal region of normal, healthy guinea pigs. The animals were observed for a period of two months and those surviving this time were sacrificed by ether anesthesia. All animals were autopsied and examinations made macroscopically for gross pathology. Acid-fast impression stains were made of the following tissues: left and right axillary lymph nodes, left and right inguinal lymph nodes, lungs, liver, spleen, and kidneys.

The dilutions of Wescodyne used and the iodine content of the respective dilutions were as follows:

Dilution of Wescodyne	Dilution in Terms of Iodine Content	Parts per Million (p.p.m.) Iodine
1:640	1:40,000	25
1:320	1:20,000	50
1:213	1:13,312	75
1:160	1:10,000	100

RESULTS

One of the animals injected with the distilled water control (two minutes) was found dead of pneumonia twelve days after injection and presented no evidence of tuberculosis. One of the two animals receiving the test suspension treated with 1:320 Wescodyne for ten minutes was also found dead at the 12th day and showed no evidence of tuberculosis. One of the two animals receiving the test suspension treated with 1:320 Wescodyne for two minutes was found dead on the 30th day following injection with no evidence of tuberculosis.

In the 1:160 treated suspension series, upon macroscopic and microscopic examinations of the two animals receiving cultures after two minutes treatment, none showed tuberculosis at the termination of the experiment. The laboratory media inoculated with the treated organisms by the latter dilution also failed to show evidence of tubercle bacilli.

All four animals in the 1:213 Wescodyne series, as well as the cultures on laboratory media, failed to

TABLE III.—WESCODYNE ACTIVITY AGAINST *B. Subtilis* SPORE-IMPREGNATED SILK SURGICAL SUTURE LOOPS[a]

Wescodyne	Experiment 1 (Loops Prepared by Stuart) Hours				Experiment 2 (Loops Prepared in Laboratory) Hours			
	2	6	10	24	2	6	8	24
(1:16)[b] 1:1,000	+[c]	+	0	0	+	0	0	0
(1:21) 1:1,331	+	+	+	0	+	0	0	0
(1:32) 1:2,000	+	+	+	0	+	+	+	+
(1:64) 1:4,000	+	+	+	+	+	+	+	+
	Minutes				Minutes			
	5	10	20	30	10	20	30	60
Resistance to constant boiling hydrochloric acid	+	+	+	+	+	+	0	0

[a] A. O. A. C. method for determining sporicidal activity of disinfectants (19).
[b] See legend *c* footnote Table I.
[c] + Indicates growth in subculture medium. 0 Indicates no growth in subculture medium.

develop tuberculosis and the cultures showed no growth.

In the 1:320 dilution group, the two animals that survived the experiment failed to show evidence of tuberculosis and the cultures were also negative.

In the 1:640 Wescodyne series, all of the animals survived the experiment and were sacrificed at that time. Both animals receiving suspensions that were exposed to the germicide for two minutes showed some evidence of tuberculosis, but growth of acid-fast organisms (4 colonies) was detected in only one of the six tubes of media. Only one of the two animals receiving the suspension that was treated by a 1:640 dilution for ten minutes showed evidence of tuberculosis. None of the media inoculated with the latter suspension showed evidence of growth.

In the distilled water control groups, the one remaining animal in the two-minute series was found dead three days before the experiment was terminated. This individual showed marked involvement of the tissues with tubercles and the cultures were also positive. A similar finding was noted in the two animals and cultures receiving the suspension which had been exposed to distilled water for two minutes.

Note may also be made of the animals in the control (water) series in which suppurative and/or caseous necrosis was observed at the site of the injection. These areas of pathology showed evidence of acid-fast organisms upon staining of the material. In the animals showing gross pathology of tuberculosis in the 1:640 dilution series, suppuration or caseation was not observed at the site of inoculation.

A final observation in the *in vivo* study was that none of the animals in the entire group, from 1:160 through 1:640, showed any evidence of a lesion or residual toxic effect at the site of injection which could be attributed to the inactivated Wescodyne.

On the basis of the *in vivo* and *in vitro* studies presented, therefore, Wescodyne in a dilution of 1:320 can be considered an effective agent for the destruction of *Mycobacterium tuberculosis*.

Sporicidal Activity of Wescodyne on *Bacillus Subtilis* Spores.—The activity of Wescodyne was determined by the A. O. A. C. method of Ortenzio, Stuart, and Friedl (19) against *B. subtilis* spore-impregnated silk suture loops made available for this study by Dr. L. S. Stuart. The test was repeated by duplicating the culturing, loop impregnation, etc., with the same strain of organisms in the author's laboratories. The results of this study are given in Table III.

From the data presented in Table III it will be noted that a concentration of 1:16 (1:1000 I_2) of Wescodyne destroyed the spores of *B. subtilis* in the loops provided by Stuart in six to ten hours. Concentration of 1:21 (1:1331 I_2) and 1:32 (1:2000 I_2) were effective in the same test between ten and twenty-four hours.

Using the same strain of organism and repeating the culturing in the author's laboratories, the 1:16 and 1:21 dilutions of Wescodyne provided sterile loops between two and six hours exposure to the germicide. The 1:32 (1:2000 I_2) concentration, on the other hand, was ineffective in this experiment even after twenty-four hours treatment.

Johns (14), using a "direct plating" method, found certain iodophors to be more effective against *B. subtilis* spores at 25 p. p. m. (1:40,000) iodine than a liquid hypochlorite (Dalglish®) at 200 p. p. m. (1:5,000) chlorine.

In a personal communication from Shapiro (20) and also from Stuart (21), certain iodophors were reported effective against pathogenic anaerobic bacilli spores (*Clostridium perfringens, Cl. tetani*) in concentrations of 500 (1:200) to 600 (1:1666) p. p. m. iodine when allowed to act on spore-impregnated surgical loops for ten to twelve hours.

DISCUSSION

Unlike most other germicides and disinfectants in use, iodine is almost equally effective against various bacteria (22), and is also active against fungi and viruses (23–25). On the basis of this wide and effective antimicrobial spectrum, iodine has been extensively used as an antiseptic for skin wounds, as a sterilizing agent for equipment and apparatus, as a prophylactic and therapeutic agent in various infectious diseases, and as a disinfectant for drinking and swimming-pool water supplies.

Considerable interest has been directed to the iodophors in which elemental iodine is solubilized by

surface-active agents, instead of iodides and alcohols. Lacking the disadvantages of aqueous and alcoholic solutions of iodine, the new formulations are stable, relatively nonstaining on fabrics and tissues, nontoxic, nonodorous, readily miscible with water, and yet retain all the desired antimicrobial activity attributed to iodine. It is probably on the basis of these added features that the California State Health Department (26) now approves the use of iodine compounds as an alternate to hot water, hypochlorites, and quaternaries for the sanitizing of restaurant eating and drinking utensils.

Allawala and Riegelman (27) prepared an excellent discussion on the properties of iodine in solutions of surface-active agents and gave an equation by which it is possible to calculate the equilibrium concentration of iodine freely dispersed in water (in contrast to micellular iodine). They also presented a correlation between the calculated concentration of iodine in water and the 99% killing time required for *Bacillus metiens* (*cereus*) spores.

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The Quantitative Measurement of *l*-Phenylephrine Hydrochloride in Dilute Aqueous Solution*

By E. L. PRATT

An optically pure specimen of levo-diacetylphenylephrine has been prepared. This substance has served as a basis for subsequent measure of the resistance of *l*-phenylephrine hydrochloride toward racemization when multiple autoclaved, and when refluxed at two pH levels. The effect of time *vs.* stereochemical stability, in commercial *l*-phenylephrine hydrochloride formulas, has likewise been considered.

THE SYMPATHOMIMETIC substance, phenylephrine hydrochloride,[1] in commercial formulas is frequently measured by methods which relate only the total of stereoisomers. One might estimate this substituted phenol by ultraviolet light absorption techniques or in terms of bromine consumption (1). The method of choice might be the photometric measure of the coupled diazotized *p*-nitroaniline reported by Auerbach (2). While these procedures are valuable measurements related to the phenolic moiety of phenylephrine, there is a need for a measurement which will relate the degree of resolution or, conversely, the extent of racemization of this substance in dilute preparations. The description and application of such a measurement is the purpose of this paper.

In general, Welsh's studies on epinephrine (3), ephedrine (4, 5), and *l*-arterenol (6) are being extended herein to phenylephrine. His published works on sympathomimetic amines present well described procedures of acetylation, prerequisites for quantitative recovery, and physical and chemical properties of the acetyl derivatives of epinephrine, ephedrine, and l-arterenol. In footnotes (7), he has reported the preparation, in quantitative yield, of the diacetyl derivative of phenylephrine but has elaborated no further

* Received March 6, 1957, from the Control Laboratories, Winthrop Laboratories, Rensselaer, N. Y.
[1] Trademark Neo-synephrine Hydrochloride.

on this subject. The preparation of acetyl derivatives of phenylephrine has likewise been reported by Bretschneider (8).

EXPERIMENTAL

Preparation of Reference Standard O³,*N*-Diacetyl-*l*-phenylephrine.—Ten grams *l*-phenylephrine hydrochloride ($[\alpha]_D^{25} = -41.2° \pm 1.8°$[2] [2% in water]) was dissolved in 500 cc. distilled water. One hundred grams of sodium bicarbonate was added to the reaction vessel followed by 50 cc. acetic anhydride. The latter substance was added in four equal portions. Reaction was permitted to continue under slight pressure until evolution of carbon dioxide ceased. The aqueous volume of reaction mixture was then extracted six times with 50-cc. portions of chloroform and the extracts were collected in a 400-cc. beaker. The chloroform was evaporated over steam. The oily residue which remained was recrystallized readily from acetone and dried at 60°. The crystalline solid was repeatedly recrystallized until the optical rotation of the derivative could be increased no further. This substance, considered as being optically pure, exhibited a specific rotation of $-115.4° \pm 1.7°$[2] (1% in $CHCl_3$). It melted sharply at 79.2–79.5° (corr.) and, in ethanol, exhibited absorption maxima at 261.5 and 269 mμ.[3] *Anal.*—Calcd. for $C_{12}H_{17}O_4N$: C, 62.14; H, 6.82; O, 25.47; N, 5.58. Found: C, 61.91; H, 6.88; O, 25.70; N, 5.65.[4]

The phenylephrine hydrochloride used to derive the diacetyl substance yielded data noted in Table I when assayed for quantitative recovery and optical purity.[5]

Using the preceding data, namely, the specific rotation of one sample of phenylephrine hydrochloride and the fraction of *l*-isomer of that same sample, as determined from its diacetyl derivative, one might readily estimate the specific rotation of an optically pure specimen of *l*-phenylephrine hydrochloride as being (−47.4).[6]

Description of Acetylation Procedure Used in Assay of Phenylephrine Hydrochloride Solutions.[7]—Transfer a volume of solution equivalent to 40 mg. phenylephrine hydrochloride to a 125-cc. separator. Add sufficient distilled water to make total of aqueous volume approximately 30-cc. (a) Extract aqueous volume of solutions containing chlorobutanol 3 times with 25-cc. portions of carbon tetrachloride. Remove chlorinated solvent as completely as possible. (b) Extract aqueous volume of solutions containing phenol 4 times with 25-cc. portions of chloroform. Remove chlorinated solvent as completely as possible. (c) Add 0.2 cc. starch T.S.,[8] to solutions containing sulfite, then, while swirling the funnel, add iodine and potassium iodide T.S. dropwise until the blue color formed persists, and immediately add just sufficient 0.1 *N* sodium thiosulfate to discharge the blue color.

Add to the liquid in the separator 2.10 Gm. of sodium bicarbonate, preventing it from coming in contact with the mouth of the separator, and swirl until most of the bicarbonate has dissolved. By means of a calibrated 1-cc. syringe, rapidly inject 1.0 cc. acetic anhydride directly into the contents of the separator. Immediately stopper the separator and shake it vigorously until the evolution of carbon dioxide has ceased. Release the pressure, as necessary, by inverting the separator and momentarily opening the stopcock. Allow separator to stand for five minutes, then extract the solution with six 25-cc. portions of chloroform. Filter each extract into a beaker through a small pledget of cotton which previously has been washed with chloroform.

Evaporate the combined chloroform extracts to about 3 cc. on a steam bath and in a current of air. Completely transfer the residue by means of small portions of chloroform to a tared 50-cc. Erlenmeyer flask and continue the evaporation until the solvent has been removed. Heat the flask and contents at 105° for thirty minutes, cool in a desiccator, and weigh the residue of diacetylphenylephrine. Completely transfer the residue with the aid of chloroform to a 5.0-cc. volumetric flask and make flask to total volume. Determine the specific rotation using a 200-mm. semimicro polarimeter tube.

Calculate the total phenylephrine hydrochloride in 1 cc. of the original sample by multiplying the weight in milligrams of the diacetyl derivative by the factor, 0.8106, and dividing the result by the number of cubic centimeters of the sample taken for the determination. Multiply the milligrams per cubic centimeter of total phenylephrine found by $(0.5 + 0.5 \times [\alpha]_D / -115.4)$ to determine the *l*-phenylephrine hydrochloride in each cubic centimeter of the original aqueous sample.

The Stability of levo-Phenylephrine Hydrochloride in Dilute Solution when Subjected to Heat.—A laboratory formulation was prepared containing in each cubic centimeter 10.0 mg. *l*-phenylephrine hydrochloride in a buffered salt solution with 0.35% phenol. The solution at pH 6.2 was filled into 20-cc. flint glass ampul sand the ampuls were sealed. The ampuls and their contents were then subjected to multiple autoclaving at 121° for thirty-minute periods. After each autoclaving period a fraction of the total ampuls was withdrawn, cooled to 25°, and the optical rotation of a representative sample was determined. In addition, an appropriate aliquot was carried through the diacetylation procedure and the resultant derivative was measured for optical activity. Table II reveals the results of these determinations.

Two 1.0-Gm. samples of phenylephrine hydrochloride were refluxed in separate 50-cc. volumes of aqueous buffer at pH 6.0 and 3.0, respectively. The buffers used were those of Clark and Lubs (10) and the time at reflux temperature was three hours

[2] 95% confidence limits.

[3] Ultraviolet spectra determined with Beckman model DK-2 spectrophotometer.

[4] The author is indebted to M. E. Auerbach of the Sterling Winthrop Research Institute for the analysis of elementary composition.

[5] Per cent optical purity = (Derivative, yield in mg., × 0.8106)/sample, mg.) × (0.5 + 0.5) × ([α]D/−115.4) × 100.

[6] [α]D Calcd. for pure *l*-phenylephrine hydrochloride = [Observed [α]D of HCl salt] × 0.5/[(*l*-isomer fraction) −0.5]

[7] Although the essentials of Welsh's quantitative acetylation procedure, as outlined for Epinephrine Solution in U. S. P. XV (9), are presented herein, application to phenylephrine solutions, of necessity, requires changes in description and in calculation.

[8] Refer to Test Solutions U. S. P. XV (pp. 1089–1102).

in each instance. After this period the solutions were cooled to room temperature and quantitatively transferred to 100-cc. volumetric flasks, which were made to total volume with distilled water and the specific rotation was determined directly and also on the diacetyl derivatives prepared from an appropriate volume of the diluted sample. The results of these measurements are reported in Table III.

The Effect of Time on the Racemization of Phenylephrine Hydrochloride in Commercial Formulas.—Samples of aqueous commercial phenylephrine hydrochloride of various ages were assayed by the diacetylation procedure. Table IV presents the data obtained in these analyses.

TABLE I.—ASSAY DATA FOR CRYSTALLINE *l*-PHENYLEPHRINE HYDROCHLORIDE

Wt., mg.	Yield, mg. Calcd.	Yield, mg. Actual	$[\alpha]_D^{25°}$ ($CHCl_3$)[a] $c = 1$	Optical Purity, %
81.0	99.93	99.8	−100.7°	93.5

[a] Diacetyl derivative.

TABLE II.—EFFECT OF MULTIPLE AUTOCLAVING ON THE OPTICAL ROTATION OF THE *l*-ISOMER OF PHENYLEPHRINE HYDROCHLORIDE IN DILUTE SOLUTION

Times Autoclaved, 15 lb. × 30 min.	$[\alpha]_D$ (20-mm. Tube) Direct Detn.	$[\alpha]_D$ ($CHCl_3$)[a] $c = 1$ (20-mm. Tube)
0	−40.5°	−99.9°
1	−40.6°	−99.9°
2	−40.8°	−100.0°
3	−43.0°	−100.4°

[a] Diacetyl derivative.

TABLE III.—EFFECT OF REFLUXING AT TWO pH LEVELS ON THE OPTICAL ROTATION OF THE *l*-ISOMER OF PHENYLEPHRINE HYDROCHLORIDE IN DILUTE SOLUTION

pH	$[\alpha]_D^{25°}$ (20-mm. Tube) Direct Detn.	$[\alpha]_D^{25°}$ ($CHCl_3$) $c = 1$ (20-mm. Tube)
6.0	−40.3°	−100.1°
3.0	−40.5°	−102.5°

DISCUSSION

From the foregoing data it may be readily noted that phenylephrine hydrochloride in aqueous formulas does not measureably racemize, when repeatedly autoclaved, or when refluxed for three-hour periods at pH levels of either 3.0 or 6.0. Commercial formulations of 0.2 and 1.0% phenylephrine hydrochloride concentration present diacetyl derivative yields in excellent agreement with label claim declarations. Because the quantity of diacetyl derivative yield is dependent on the phenolic moiety, as well as the terminal secondary amine, these data further suggest that there is no significant chemical degradation in the commercial phenylephrine hydrochloride preparations assayed. With respect to the levo potency findings in solutions manufactured within the seven-year period sampled, the data of Table IV indicate that racemization has not occurred, and that the quantity of levo-phenylephrine hydrochloride found was dependent only on the degree of initial resolution of the crystalline hydrochloride salt used in solution preparation.

In instances where solutions are 1% in phenylephrine hydrochloride concentration, a direct measure of optical rotation is possible. The diacetyl derivative, however, presents an angular measurement of greater than twice the magnitude of that of an equal weight of phenylephrine hydrochloride. Hence, a corresponding increase in measurement precision is realized when the diacetyl procedure is used. There is also afforded in the diacetyl procedure a measure of the total of stereoisomers without the need of an independent assay. Solutions of less than 1% phenylephrine hydrochloride content cause angular readings too minute to be practical for measurement purposes.

SUMMARY

The substance *l*-diacetylphenylephrine has been prepared and resolved to that which is believed to be an optically pure specimen. This substance has been used as a reference compound in the subsequent measure of levo potency in phenylephrine hydrochloride solutions. The quantitative aspects of this measure have been

TABLE IV.—ANALYSES OF AGED COMMERCIAL PHENYLEPHRINE HYDROCHLORIDE SOLUTIONS VIA DIACETYLATION PROCEDURE

Age, Yr.	Label Claim, mg./cc.	Levo Isomer Found %	Diacetyl Der. Obtained, %	$[\alpha]_D$ Estimated[a]	$[\alpha]_D$ Reported[b]
7	2.0	81.5	100.2	−29.9°	−28.7°
7	2.0	83.5	103.7	−31.8°	−28.7°
7	10.0	82.7	100.3	−31.0°	−28.7°
6.75	10.0	89.5	99.1	−37.4°	−36.6°
6.75	10.0	88.6	99.3	−36.6°	−36.6°
6.5	10.0	89.6	98.9	−37.5°	−36.6°
6.5	10.0	90.1	99.5	−38.0°	−36.6°
6.25	10.0	91.9	101.1	−39.7°	−36.6°
0.25	10.0	93.2	99.7	−41.0°	−40.7°
0.2	10.0	92.0	99.5	−39.8°	−40.7°

[a] Crystalline phenylephrine hydrochloride estimated from determination of optical potency. Assuming no racemization has occurred the specific rotation of the crystalline phenylephrine hydrochloride used in solution preparation may be estimated from the expression: $[\alpha]_D$ of powder = *l*-isomer, % obtained by the diacetyl procedure × (−0.948) + 47.4.

[b] Determined for crystalline phenylephrine hydrochloride prior to preparation of solution.

demonstrated. Calculation of the specific rotation of optically pure *l*-phenylephrine hydrochloride has been made from the data of the resolved *l*-diacetyl substance. The resistance of phenylephrine hydrochloride toward racemization in a simulated formulation after repeated autoclaving has been demonstrated, as has the resistance of phenylephrine hydrochloride in solutions buffered at pH 3.0 and 6.0 when refluxed for three-hour periods.

Analytical procedure applicable to commercial formulas has been described and its use in measurements on aged commercial solutions has been demonstrated. The results of the analyses made at this time indicate racemization has not occurred and that the levo- isomer content is only dependent on the degree of initial resolution of a given crystalline phenylephrine hydrochloride.

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Notes

A Note on the Root Bark Alkaloids from *Alstonia Constricta* F. Muell.*

By GORDON H. SVOBODA†

WHILE THE ALKALOIDS from the trunk bark of *Alstonia constricta* F. Muell. have been quite exhaustively studied in the past, little investigation has been conducted on the alkaloids contained in the root bark. The presence of reserpine in *A. constricta* root bark was recently reported by Crow and Greet (1), and laboratory results were published by Curtis, Handley, and Somers (2).

A. constricta is a small, often bushy tree, 10–30 feet tall, and its distribution is confined to northeastern Australia. All parts of the plant are extremely bitter, and the inner bark of the stem and roots is a golden yellow. The root bark is relatively thick, being approximately one inch thick on roots four inches in diameter. It is very succulent and loses 80% or more of water when dried.

A sample of root bark from New South Wales yielded reserpine (0.05%), tetrahydroalstonine, isolated as the maleate salt, and a third weak base (0.11%), which is apparently the alstonidine of Hesse (3). α-Yohimbine (rauwolscine) was isolated as the hydrochloride from the moderately strong base fraction by a chromatographic procedure. Inasmuch as the purpose of this investigation was to ascertain the feasibility of commercial utilization of *A. constricta* root bark as a source of reserpine, little was done with the strong base fraction other than to establish the presence of alstonine by paper chromatography. Should the sample examined be characteristic of the root bark generally available, it cannot at this time be considered as an economical source of reserpine.

* Received February 18, 1957, from the Lilly Development Laboratories, Eli Lilly and Co., Indianapolis, Ind.
† The author is grateful to Mr. A. T. Oliver for laboratory assistance, to Dr. E. W. Grant for paper chromatographic studies, and to Mr. W. L. Brown, Miss G. Beckman, Mr. H. L. Hunter, and Mr. G. M. Maciak for microanalytical data.

EXPERIMENTAL

Dried ground root bark (3,178 Gm.) was intimately mixed with 2,100 cc. of 2% tartaric acid solution, covered with extraction naphtha, and allowed to macerate for eighteen hours. The presence of the tartaric acid served to bind the stronger bases in the drug, thus affording an initial separation of weak and strong bases during extraction. The drug was drained of extraction naphtha, was covered with benzene, and was allowed to macerate for two hours. Approximately 32 liters of percolate was collected during six hours and this percolate was concentrated *in vacuo* to 4,000 cc. As a means of further purification from extraction naphtha-insoluble, benzene-soluble impurities, the concentrated percolate was vacuum steam-distilled with one 4,000-cc. and two 2,000-cc. portions of 2% tartaric acid.

The tartaric acid solution was extracted with two 4,000-cc. portions of ethylene dichloride. The light yellow residue obtained from this extract yielded 1.6 Gm. (0.05%) of reserpine from 40 cc. of hot methanol. The paper chromatogram indicated a single component and the X-ray pattern was identical to standard reserpine prepared in our laboratory from *Rauwolfia vomitoria* Afz.

The reserpine mother liquor was concentrated *in vacuo* to dryness. The residue was extracted with

two 500-cc. portions of ether, and the extract was concentrated *in vacuo* to dryness. The residue was dissolved in a small volume of chloroform, and the solution was chromatographed on a column of 200 Gm. of acid-washed aluminum oxide. A light amber-colored fraction, displaying a green fluorescence, was collected, and an amorphous maleate was precipitated by the addition of an ether solution of maleic acid to an ether solution of the chloroform-free residue of this fraction. The resulting dry, amorphous maleate lent itself readily to crystallization from methanol. Recrystallization of this maleate from methanol yielded 0.2 Gm. of a colorless salt. The infrared absorption spectrum of the base extracted from the salt into chloroform showed it to be tetrahydroalstonine.[1]

The extracted acid water exhibited an intense blue fluorescence. It was made alkaline to litmus by the addition of 28% ammonia and was extracted with one 4,000-cc. portion of ethylene dichloride. The golden yellow residue obtained from the extract yielded 3.5 Gm. (0.11%) of colorless crystals, m. p. 183–185°. In all probability this was alstonidine. It was readily soluble in 5% hydrochloric acid, in which it displayed a blue fluorescence. The same fluorescence was shown by an alcohol solution of the base and was intensified by the addition of water. The base was readily recrystallized from acetone. Slow crystallization from ether produced crystals large enough for single crystal X-ray studies;[2] m. p. 186–188°; allowing the melt to solidify and then remelting gave m. p. 185–187°.

Anal.—Calcd. for $C_{20}H_{24}N_2O_4$: C, 69.45; H, 6.36; N, 7.36; O, 16.82; (C) CH_3, 3.95; —OCH_3, 8.16; (N) CH_3, 3.90. Found: C, 69.61; H, 6.59; N, 7.21; O, 17.03; (C) CH_3, 3.60; —OCH_3, 8.09; (N) CH_3, 3.72. Physical data corresponded to that obtained on a sample of "Alstonidine" previously submitted to our laboratory by Dr. R. C. Elderfield.[3]

After the acid benzene extract had been collected, gaseous NH_3 was bubbled through the drug until it was alkaline, during which time the drug swelled to about double its original volume. Approximately 32 liters of percolate was collected, this being concentrated *in vacuo* to about 4,000 cc. Extraction with two 4,000-cc. portions of 2% tartaric acid, neutralization of the acid extract with 28% ammonia, and extraction with five 4,000-cc. portions of ethylene dichloride yielded 67 Gm. of reddish-colored amorphous material. The residue yielded no crystalline material from the usual organic solvents. It was extracted with ether, in which it was only slightly soluble, the solution possessing a brilliant green fluorescence. After having been dried over sodium sulfate, the ether was removed *in vacuo*, yielding 7.2 Gm. of a light yellow residue. Paper chromatography in a system of butanol–water showed two components consisting of a small amount of alstonine and a large amount of a slightly faster-moving alkaloid.

The amorphous residue was dissolved in a small volume of chloroform and was passed through a column containing 200 Gm. of acid-washed aluminum oxide. A light yellow fraction possessing a light blue fluorescence was collected. The chloroform was removed *in vacuo*, the residue was dissolved in ethanol, and gaseous HCl was passed through the solution. The crystals which deposited after having been chilled for almost two weeks were identified by the infrared spectrum of the base as α-yohimbine (rauwolscine).[4]

The drug was finally extracted with alcohol. The concentrated alcohol percolate was then extracted with 2% tartaric acid solution. Ethylene dichloride extraction of the alkalinized extract yielded 23.7 Gm. of a dark amber, amorphous residue. Paper chromatography of this material showed the presence of a large amount of alstonine. Further work was not attempted.

[1] Authentic sample kindly furnished by Dr. R. C. Elderfield, University of Michigan.

[2] These data, along with other physical data and their interpretation, appear as two separate notes in THIS JOURNAL.

[3] We thank Dr. R. C. Elderfield, University of Michigan, for submission of a sample labeled "Alstonidine."

[4] Sample kindly supplied by Dr. A. Chatterjee, University College of Science and Technology, Calcutta, India.

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A Note on the Crystallography of Alstonidine*

By ANN VAN CAMP and HARRY A. ROSE

The alkaloid alstonidine has been extracted from the rootbark of *Alstonia constricta* as described by Svoboda (1). A discussion of the chemistry of this compound is given by Boaz (2).

* Received February 18, 1957, from Eli Lilly and Company, Indianapolis 6, Ind.

The authors wish to thank Dr. G. H. Svoboda of these laboratories for providing the crystals from which these data were obtained.

A sample submitted earlier to these laboratories by Dr. R. C. Elderfield of the University of Michigan was found to be the same compound by comparison of the X-ray powder data.

EXPERIMENTAL

Crystals suitable for X-ray and optical studies were obtained by crystallization from ether. The crystals were orthorhombic blades elongated parallel to the *c* axis.

The X-ray powder diffraction data were obtained

using chromium radiation and vanadium filter with a camera 114.6 mm. in diameter. A wavelength value of 2.2896 A was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around the *b* and *c* axes.

On heating, alstonidine melts in the range 186–188° as determined on a Kofler hot stage.

TABLE I.—X-RAY DIFFRACTION DATA FOR ALSTONIDINE

Unit cell dimensions: $a_0 = 17.64$ A, $b_0 = 21.40$ A, $c_0 = 5.20$ A
Formula weights per cell: 4
Formula weight: 380.4
Density: 1.288 Gm./cc. (flotation), 1.289 Gm./cc. (X-ray)
Axial ratio: $a:b:c = 0.8243:1:0.2430$
Space group: $P2_12_12_1$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA FOR ALSTONIDINE

Refractive indices: (5893 A, 25°) $\alpha = 1.590$, $\beta = 1.688$, $\gamma = 1.692$
Optic axial angle: (−) 32° (calcd. from α, β, and γ)
Acute bisectrix: $\alpha = c$
Optic axial plane: 010

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	*h k l*	*d* (Calcd.)
10.73	0.40	020	10.70
8.82	1.00	200	8.82
8.17	0.40	210	8.15
6.82	0.20	220	6.81
6.64	0.20	130	6.61
5.68	0.10	310	5.67
5.57	0.10	230	5.55
5.37	0.30	040	5.35
5.07	0.80b	140, 011	5.12, 5.05
4.90	0.05	111	4.86
4.68	0.10	021	4.67
4.54	0.50	240, 330, 121	4.57, 4.54, 4.52
4.13	0.80	221	4.13
3.79	0.80	231	3.79
3.65	0.30	321, 141	3.66, 3.65
3.56	0.05	060	3.57
3.44	0.20	241	3.43
3.32	0.10	260	3.31
3.16	0.10	530	3.16
3.09	0.10	450	3.07
2.94	0.05	600	2.94
2.89	0.10	270	2.89
2.69	0.05	080	2.68
2.65	0.05	180	2.65
2.43	0.05	..	..
2.25	0.05	..	..
2.08	0.05	..	..

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Alstonia Alkaloids VII. The Structure of Alstonidine*

By HAROLD BOAZ, ROBERT C. ELDERFIELD, and ERHARD SCHENKER

ISOLATION OF ALSTONIDINE has been reported by several investigators (1, 2). Van Camp and Rose (3) have given X-ray data and a molecular weight determination. We now wish to present some physical and chemical data and their structural implications.

Titration in 66% dimethylformamide gives a pK_a' of 5.95 compared to 6.10 for harman in the same solvent. The molecular weight by titration is 382.5; by X-ray unit cell dimensions and density (3), 380.1; calculated for $C_{22}H_{24}N_2O_4$ (2), 380.44.

The ultraviolet spectra of alstonidine, of harman[1] and indole-N-methyl harman[2] are shown in Figs. 1 and 2. These spectra demonstrate the presence of a methyl substitution on the indole nitrogen of alstonidine. They eliminate any oxygen substitution on the harman portion of the molecule. The higher wavelength observed in the spectrum of alstonidine as compared to those of the harmans for the maximum near 300 mμ is reasonable for the effect of exchanging the C-methyl group in the harmans with a heavier group in alstonidine (4).

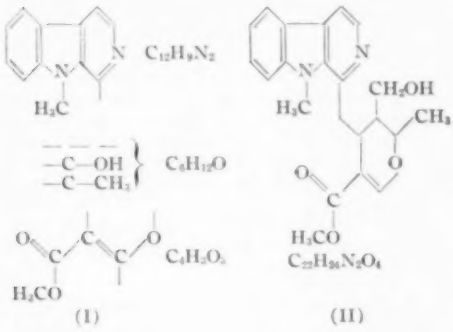
The contribution of an ester conjugated with a vinyl ether is evident in the ultraviolet spectra at 235 mμ and in the infrared spectrum in chloroform solution (Fig. 3) at 5.89 μ and 6.14 μ. These wavelengths are within experimental error the same for the corresponding bands of tetrahydroalstonine.

Other assignments of bands in the infrared spectrum include the following: 3.18 μ, hydrogen bonded hydroxyl; 6.16, 6.39, 6.74, and 7.13 μ, indole-N-methyl harman.

Group analysis (2) shows an N-methyl as well as

* Received February 18, 1957, from the Lilly Research Laboratories and the Department of Chemistry, University of Michigan. Alstonidine has been isolated in both laboratories.
The work here reported was done in part with the aid of Research Grant H-1733 from the National Institutes of Health to the University of Michigan.
[1] Synthetic sample kindly supplied by Dr. G. B. Kline.
[2] Synthetic sample kindly supplied by Dr. Carl Djerassi.

a methoxyl and a C-methyl. The above observations together with elemental analysis (2) establish the partial structure I.



Structure II is supported by the following observations and by analogy with known structures such as tetrahydroalstonine. The infrared spectrum of the base in chloroform over the concentration range 0.0075 *M* to 0.034 *M* shows that the molar absorptivity of the band arising from the stretching of a strongly hydrogen bonded hydroxyl group is independent of concentration. The band maximum occurs at 3.18 μ. A similar observation was made with the hydrochloride salt except that the band maximum is at 3.00 μ. The free hydroxyl band at 2.79 μ is very weak in both spectra. Its molar absorptivity is also independent of concentration.

Unless for both molecules a dimer is very stable in chloroform (not likely for the positive ion) these observations argue strongly for an intramolecular involvement of the hydroxyl. The stronger hydrogen bond (3.18 μ) is probably directed largely to the basic nitrogen. The weaker hydrogen bond (3.00 μ) in the ion is accompanied by greater changes in the spectrum between 9.0 and 9.5 μ than at the carbonyl band on salt formation suggesting hydrogen bonding of the hydroxyl to the ether oxygen. A molecular model of II illustrates the ease of formation of such a hydrogen bond and the difficulty of forming a hydrogen bond with the ester carbonyl.

Additional evidence for II as representative of alstonidine is provided by certain reactions undergone by the alkaloid. Alkaline hydrolysis results in the formation of alstonidinic acid, isolated as the trihydrate. Reesterification of alstonidinic acid with diazomethane gave alstonidine. The presence of an acylatable hydroxyl group was demonstrated by the formation of an acetyl derivative which was also isolated as a trihydrate.

EXPERIMENTAL[3, 4]

Hydrolysis of Alstonidine. Alstonidinic Acid.—A solution of 150 mg. of alstonidine in 7.5 ml. of ethanol and 7.5 ml. of 1 *N* sodium hydroxide solution was refluxed for five hours and allowed to stand at room temperature for fifteen hours. After removal of the ethanol at reduced pressure the pH of the solution was adjusted to 3 with 2 *N* hydrochloric acid. On further concentration of the solution under reduced pressure alstonidinic acid crystallized as light yellow needles. After two recrystallizations from methanol–ethyl acetate, the acid (82 mg.) melted at 238–240° (decompn.). The analytical data indicated retention of three waters of crystallization

[3] Melting points are corrected for stem exposure.
[4] Microanalyses by Spang Microanalytical Laboratory, Ann Arbor, Mich.

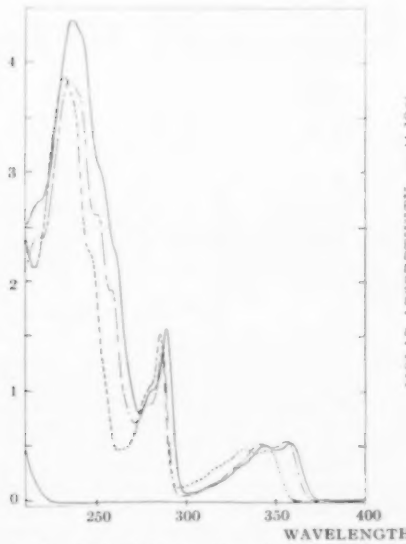


Fig. 1.—Ultraviolet spectra of —— alstonidine, · · · harman and — · — indole-N-methyl harman in methanol at 0.05 *M* potassium hydroxide.

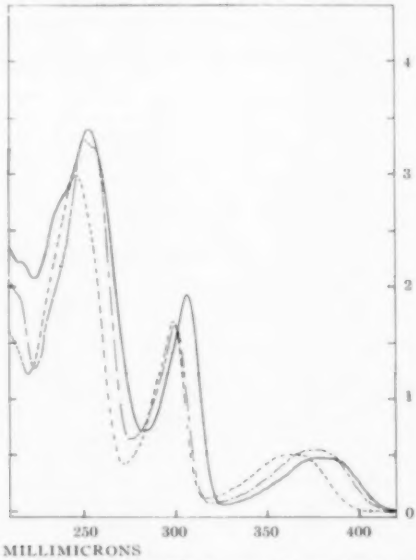


Fig. 2.—Same as Fig. 1 except in methanol at 0.05 *M* hydrochloric acid. Ultraviolet spectra were obtained on a Cary Model 14 spectrophotometer.

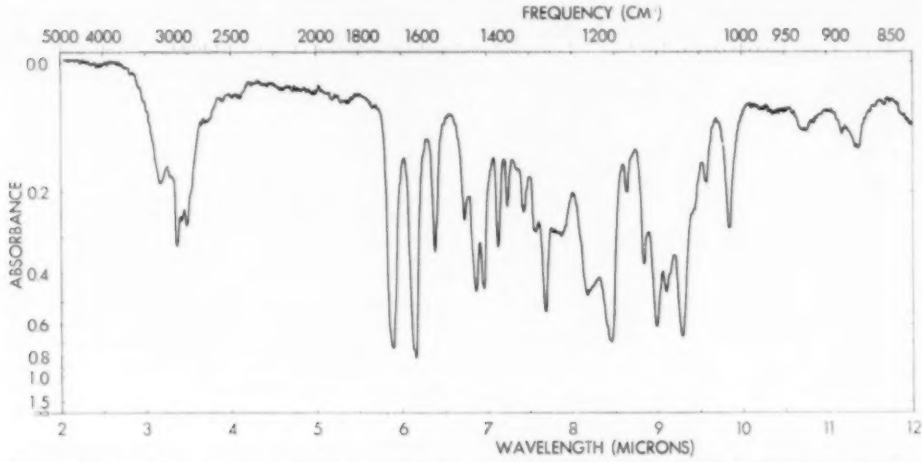


Fig. 3.—Infrared spectrum of alstonidine in chloroform; 0.145 *M* in 0.11 mm. path; Perkin-Elmer Model 21 spectrophotometer.

even after drying at 1 mm. over phosphorus pentoxide. No ionic chlorine was detectable with silver nitrate.

Anal. Calcd. for $C_{21}H_{22}N_2O_4 \cdot 3H_2O$: C, 59.99; H, 6.67; N, 6.66. Found: C, 60.08; H, 6.71; N, 6.65.

Reesterification of Alstonidinic Acid.—To a solution of 30 mg. of alstonidinic acid in 5 ml. of methanol an excess of an ethereal solution of diazomethane was added. After standing for thirty minutes at 0° the ether was removed under reduced pressure. Dilution of the resulting methanol solution and further concentration gave 29 mg. of alstonidine as fine colorless needles, m. p. and mixture m. p. with an authentic sample, 188–190°. The infrared spectrum was identical with that of authentic alstonidine.

Acetylation of Alstonidine.—A solution of 50 mg. of alstonidine and 1 ml. of acetic anhydride in 5 ml. of anhydrous pyridine was allowed to stand at room temperature for three days. After addition of 1 ml. of ethanol the solution was taken to dryness under reduced pressure at 50°. Recrystallization of the residue from aqueous acetone gave acetylalstonidine as fine needles, m. p. 92–96°. The analytical data indicated retention of three waters of crystallization even after drying over phosphorus pentoxide at 1 mm.

Anal. Calcd. for $C_{23}H_{28}N_2O_5 \cdot 3H_2O$: C, 62.87; H, 6.60; N, 6.12. Found: C, 63.22; H, 6.68; N, 6.59.

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A Note on the Assay of Zinc Stearate*

By JAN S. FABER†

THE METHOD OF ANALYSIS for zinc stearate in USP XV is in fact a determination of the fatty acids from the result of which the equivalent of zinc oxide is calculated. This method is accurate only when the zinc stearate contains equivalent proportions of zinc and of the fatty acids. Several samples, containing sodium stearate as contaminant, gave us false high results for zinc oxide, when the USP. method was used. A complexometric determination combined with the USP method, however, gave good results for both the fatty acids and the zinc content. The same method can be applied to the assay of magnesium stearate USP.

The official zinc stearate does not consist of pure $Zn(C_{17}H_{35}COO)_2$, but is described as a compound of zinc with variable proportions of stearic and palmitic acid, containing the equivalent of not less than 13 per cent and not more than 15.5 per cent of ZnO (1).

The principle of the USP method for determining the percentage of zinc oxide involves boiling

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† The author wishes to express his sincere thanks to Miss G. Hazeveld for her help in the performance of the analyses.

the zinc stearate with an excess of 0.1 *N* sulfuric acid, collecting the stearic and palmitic acids on a filter, and washing the precipitate with water until neutral to litmus. The combined filtrates are then titrated with 0.1 *N* sodium hydroxide to determine the excess of sulfuric acid.

When the zinc stearate is contaminated with sodium stearate, as it is in some commercial samples, the USP method can give results, within the official limits, although the zinc content is in fact somewhat lower. The amount of sulfuric acid consumed, indicates the equivalent of the sum of the zinc and sodium content (2) instead of the zinc actually present.

It is possible to combine the USP assay method with a complexometric zinc determination by titrating the neutralized filtrate with 0.05 *M* disodium ethylenediaminetetraacetate (disodium Versenate®) at a pH of 10, using eriochrome black T as the indicator, and thus determine the actual percentage of zinc. When zinc stearate is pure, both titrations give the same ZnO equivalent.

TABLE I.—ZINC OXIDE DETERMINATIONS IN TWO COMMERCIAL SAMPLES OF ZINC STEARATE BY FOUR DIFFERENT METHODS

	Complexometric Procedure I, ZnO, %	Complexometric Procedure II, ZnO, %	U. S. P. XV Method, ZnO, %	Complexometric Procedure III, ZnO, %
Sample 1[a]	7.99[c] ± 0.045	8.05[c] ± 0.149	13.46	8.05
			13.50	8.06
Sample 2[b]	12.96[c] ± 0.025	... ...	14.41	13.01
			14.43	13.04

[a] High sodium stearate content. [b] Lower sodium stearate content. [c] Mean of 6 determinations.

TABLE II.—ZINC OXIDE DETERMINATION ON ZINC STEARATE CONTAINING ADDED SODIUM STEARATE

Sodium Stearate Added, %	USP XV ZnO, %	Complexometric Procedure III ZnO, %
0	14.39	13.06
	14.36	13.03
	14.39	13.01
	14.39	13.03
5	14.40	12.38
	14.45	12.42
	14.43	12.42
	14.44	12.42
10	14.41	11.84
	14.39	12.14
	14.41	12.11
	14.41	11.85

EXPERIMENTAL

Reagents and Volumetric Solutions

Disodium Ethylenediaminetetraacetate, 0.05 Molar.—Dissolve 18.605 Gm. of the disodium salt of ethylenediaminetetraacetic acid, $Na_2H_2(C_{10}H_{12}O_8N_2)\cdot 2H_2O$, of reagent purity, (disodium Versenate®, Complexon® III) in water by warming, and dilute to 1000 cc. The pH of this solution must be about 4.7.

Zinc Sulfate, 0.05 Molar.—Dissolve 14.38 Gm. of reagent grade zinc sulfate, $ZnSO_4\cdot 7H_2O$, in sufficient water to make 1000 cc.

Ammonia-Ammonium Chloride Buffer (pH 10).—Mix 70 Gm. of ammonium chloride, 460 ml. of stronger ammonia water (25 per cent NH_3), and a sufficient quantity of water to make 1000 cc.

Eriochrome Black T Indicator.—A mixture of 1-(1-hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sodium sulfonate and sodium chloride in the ratio 1:100. Keep in a tight container protected from light.

Reagents not mentioned here are the same as indicated in the USP.

Procedures

Procedure I: Indirect Complexometric Titration of Zinc Stearate (3).—Dissolve about 0.5 Gm. of zinc stearate, accurately weighed, in 50 ml. of absolute ethanol and 1 cc. of hydrochloric acid. Add 25 cc. of 0.05 *M* disodium ethylenediaminetetraacetate, then, dropwise, about 1.2 cc. of diluted ammonia solution (10 per cent NH_3) until a precipitate begins to form (pH about 10). Add 5 cc. of ammonia-ammonium chloride buffer solution and heat to above 50°. Add about 0.04 Gm. of eriochrome black T indicator and titrate with 0.05 *M* zinc sulfate until the first color change from blue to red-violet. Each cc. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 0.004069 Gm. ZnO.

Procedure II. Indirect Complexometric Titration after Ignition of the Stearate.—Transfer about 1 Gm. of zinc stearate to a large, tared procelain crucible and weigh accurately. Heat at first very gently, but more strongly after melting. Ignite and transfer the residue into a flask with the aid of 2 cc. of diluted hydrochloric acid and some water. Boil and add 50 cc. of 0.05 *M* disodium ethylenediaminetetraacetate, 4 to 8 cc. of 1 *N* sodium hydroxide, 5 cc. of ammonia-ammonium chloride buffer solution, and about 0.03 Gm. of eriochrome black T indicator. Titrate with 0.05 *M* zinc sulfate until the first color change from blue to red-violet.

Procedure III: Complexometric Titration Following the U. S. P. Acidimetric Titration.—Boil about 1 Gm. of zinc stearate, accurately weighed, with exactly 50 cc. of 0.1 *N* sulfuric acid as indicated in the USP, complete the assay, and calculate the ZnO content. Add to the solution, 5 cc. of ammonia-ammonium chloride buffer solution and about 0.05 Gm of eriochrome black T indicator. Heat the solution to about 40° and titrate with 0.05 *M* disodium ethylenediaminetetraacetate until the color has fully changed from red-violet to blue.

DISCUSSION

Procedure II is less suitable than Procedure I. An inherent source of error in Procedure II is due to the fact that stearic acid is flammable even when heated gently, and unless unusual care is exercised loss may occur. The color change is less sharp than in Procedure I.

In Procedure I sudden and uncontrolled boiling sometimes occurs when dissolving zinc stearate in alcohol. If this is not guarded against, it may result in loss of the sample. During the titration the temperature of the solution should be above 50°, because otherwise a precipitate will be formed. Even if this happens, the color change remains fairly sharp.

Procedure III, combining the complexometric with the U. S. P. procedure, is the method of choice for pharmacopeial purposes. It is necessary to add more than the usual amount of the eriochrome black T indicator, because of the presence of the methyl orange from the acidimetric titration. Although sodium is the most frequent contaminant of zinc stearate, alkaline earths may also be present and titrated together with the zinc in the complexometric procedures. It is, however, possible to determine the alkaline earths first. By adding potassium cyanide to a solution containing zinc and an alkaline earth, zinc ions are masked by complexing them with cyanide ions. Only the alkaline earth ions can now be titrated with disodium ethylenediaminetetraacetate. When the alkaline earths have thus been determined, the zinc cyanide complex is decomposed by formaldehyde solution and the free zinc ions are titrated with a second portion of disodium ethylenediaminetetraacetate (4).

High contamination with alkalies and earths can be detected by the U. S. P. limit test (1). Commercial samples, not complying with this limit test, would give too high a figure for ZnO in the U. S. P. assay, but when completing this assay with a complexometric titration as indicated in procedure III, the actual amount of ZnO will be determined. This also holds good for samples containing free fatty acids, and the same method can be applied to magnesium stearate.

SUMMARY

Three complexometric procedures for the zinc determination in zinc stearate have been described and discussed. It has been pointed out that the assay in the U. S. P. XV from which a ZnO percentage is calculated, is in fact a determination of fatty acids. The percentage of ZnO may only be calculated when the ratio Zn to fatty acids is in accordance with the chemical formula. For commercial samples containing sodium stearate, a complexometric titration is recommended to complete the U. S. P. method.

The results obtained with samples contaminated with sodium stearate show that the calculation of the zinc oxide equivalent by the U. S. P. method is not accurate. In such samples the difference between the zinc oxide determination by the U. S. P. method and that obtained by the complexometric procedure gives the contamination quantitatively while the complexometric method gives the correct figures for the zinc oxide percentage. The method can also be applied to magnesium stearate.

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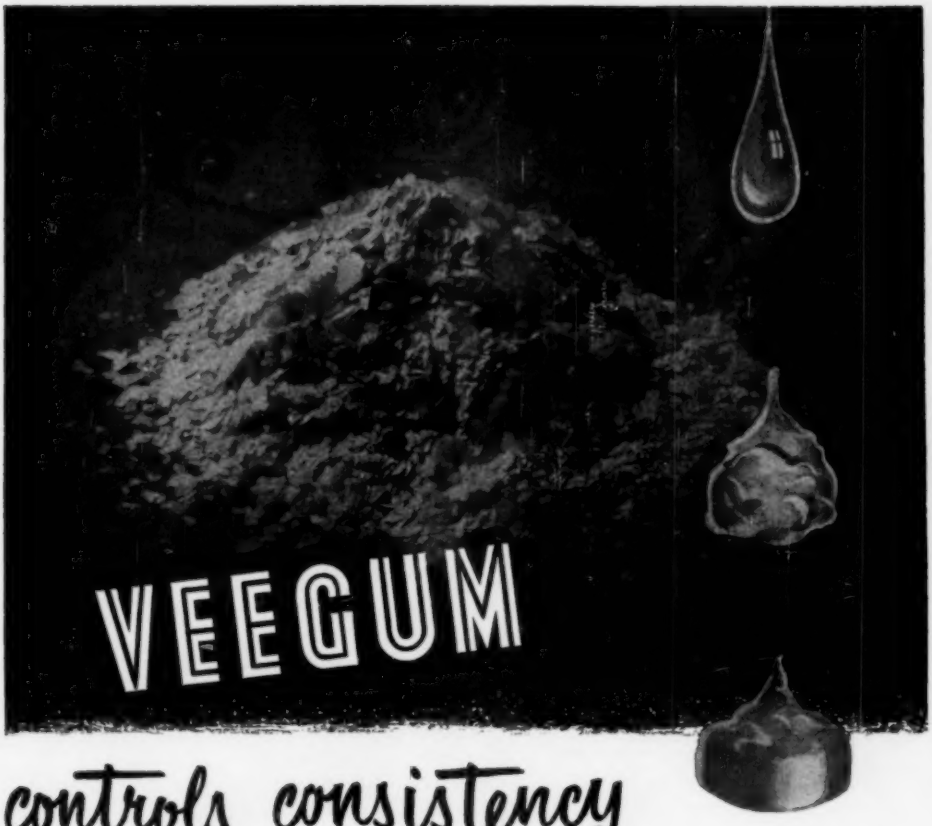

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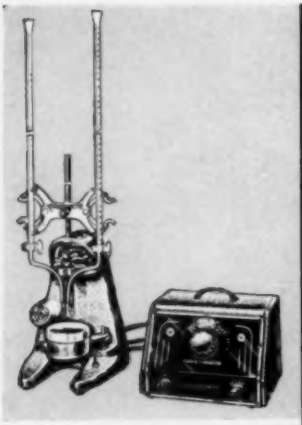

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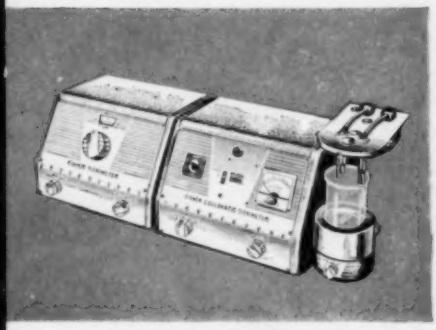

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